Title	Development of Analytical Technology to Improve the Ability to Detect and Identify Toxic Substances Used in Terrorism and Crime
Author(s)	Yamaguchi, Akinori; 山口, 晃巨
Degree Grantor	北海道大学
Degree Name	博士(工学)
Dissertation Number	甲第14920号
Issue Date	2022-03-24
DOI	https://doi.org/10.14943/doctoral.k14920
Doc URL	https://hdl.handle.net/2115/85292
Type	doctoral thesis
File Information	YAMAGUCHI_Akinori.pdf

Doctor Thesis

Development of analytical technology to improve the ability to detect and identify toxic substances used in terrorism and crime

（テロや犯罪で使用される有毒物質の検知・同定能力向上のための分析技術の開発）

Hokkaido University Graduate School of Chemical Sciences and Engineering

Bioanalytical Chemistry Laboratory

Akinori Yamaguchi

Instructor: Prof. Manabu Tokeshi

2022

Contents

Publications

1. A Screening Method for Cyanide in Blood by Dimethoxytriazinyl Derivatization–GC/MS
   Yamaguchi, A.; Miyaguchi, H. *J. Chromatogr. Sci.* **2021**. *59*, 1–6.
2. Paper-Based Analytical Device for the On-Site Detection of Nerve Agents
   Yamaguchi, A.; Miyaguchi, H.; Ishida, A.; Tokeshi, M. *ACS Appl. Bio Mater.* **2021**, *4*, 6512–6518.
3. Analysis of Novichok A-series Degradation Products in Human Urine by Dimethoxytriadinylation LC–MS/MS
   Yamaguchi, A.; Miyaguchi, H.; Ishida, A.; Tokeshi, M. *Anal. Chem.* accepted.

Chapter 1

Introduction: Police, Poison, and Chemical Warfare Agents

1. Introduction

1-1. Chemical detections and analyses as police activities

In large-scale poisoning cases, multiple persons are poisoned at once and the causative compound of the poisoning is often unknown, resulting in a major social problem. Police and other first responders rush to the scene of the poisoning, rescue the victims, and start an investigation if such a case is suspected, and in this consequence management, prompt identification of the poisoning-causing substance by on-site detection is required. The forensic science laboratory of the prefectural police headquarters is in charge of the analysis of poisonous and harmful substances involved in crimes, and the results of these analyses become scientific evidence to prove the crime in incident management. In addition, the results of these analyses play a decisive role in the direction of the initial investigation in the consequence management of poisonous and harmful substance cases, and are essential in determining the treatment plan for victims.

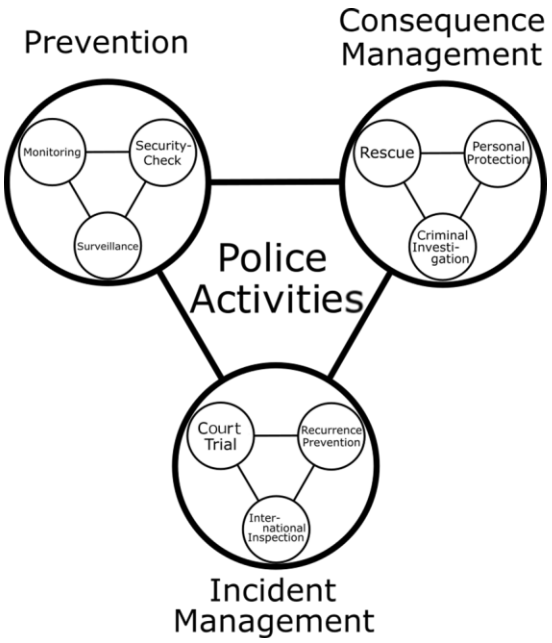


Figure 1-1. Police activities and objectives in poisoning and terrorism cases.

The chemistry division of the police force has a mission to respond to criminal cases, and it is engaged in forensic investigation and forensic science technology development related to chemistry.[1] On the other hand, there is a security division whose mission is to deal with terrorist incidents and large-scale accidents. Figure 1-1 relates the concepts and objectives of response to poisoning incidents, terrorist attacks, and accidents in which toxic compounds are present.[2] In the "prevention" phase, chemical warfare agents are monitored from a defensive standpoint in public places, border areas, airports, large-scale event sites, critical facilities, and chemical weapons processing facilities. In the "consequence management" phase, which is generally within one day of the occurrence of a terrorist attack or mass poisoning, initial response teams (police task force, firefighting and rescue teams, Coast Guard) that come to the scene will conduct on-site detections for the purposes of personal protection and damage minimization. The samples collected at the scene are transported to specialized laboratories for analysis to contribute information to the initial investigation and identification of hazardous and poisonous substances which in turn contributes to emergency rescue. In "incident management," more accurate analysis is conducted, mainly by instrumental analysis, in order to submit evidence supporting the guilt of the suspect to the trial court.[3]

Figure 1-2. Toxicological analysis in terrorism cases and crime incidents.

Since it takes a long time to deliver field samples to specialized laboratories, it is essential to focus on field detection in order to minimize damage (Figure 1-2). In the 20 years since 9/11, various efforts have been made to protect the public, but the threat of chemical warfare agents was reaffirmed in 2013 during the Syrian civil war, when sarin gas was used by government forces.[4] As with forensic investigations, it is necessary to approach field operations objectively and rationally from a chemical perspective.

In laboratory analysis (Figure 1-2), screening pre-testing of field samples and victims' biological samples, pre-treatment, and instrumental analysis centered on gas chromatography-mass spectrometry (GC–MS) and liquid chromatography-mass spectrometry (LC–MS) are used for rapid detection and identification of causative agents. Since chemical warfare agents are easily degradable, it becomes difficult to detect the agents themselves in evidence samples over time, and instead, detection of their metabolites and degradation products becomes effective. Related compounds such as synthetic intermediates and by-products of chemical warfare agents are also effective indicators of the presence of chemical warfare agents. Instrumental analysis can be used not only as a highly sensitive detection and identification method for chemical warfare agents, but also as a method to prove exposure to chemical warfare agents by detecting adducts with biological target macromolecules (e.g., enzymes). On the other hand, for monitoring and on-site detection of hazardous materials, microfluidic devices and sensor technologies contribute to the rapid and simple detection using portable or installed detectors.

1-1-1. Poisons

1-1-1-1. Overview of toxicological analysis

The Matsumoto and Tokyo subway sarin gas incidents in the mid-1990s, as well as the 1998 chain of poisoning incidents that followed the Wakayama poisoned curry case, exposed the inadequacy of coordination and information transfer among local administrative agencies (fire departments, emergency services, public health centers, police, etc.), and thus the inadequacy of Japan's crisis management system. Therefore, after the 1998 incidents, the Cabinet Secretariat and other relevant ministries (National Police Agency, Ministry of Health, Labour and Welfare, Fire and Disaster Management Agency, Ministry of Defense, etc.) and related experts worked to establish a crisis management system to deal with poisoning incidents[5,6,7,8]. Currently, in the event of a poisoning case of unknown origin or contamination of food and beverages with foreign substances, regardless of whether or not there is criminal involvement, biological samples and crime scene samples derived from the victims as evidence samples are promptly sent to the responsible prefectural police forensic science laboratory. The response system is maintained at the local administrative level. Information on the crime scene investigation (police headquarters) and information on the victims’ poisoning symptoms (e.g., emergency medical center) are necessary to improve the efficiency of the forensic toxicology examination. On the other hand, information on the detection of poisoning-causing substances obtained by the forensic science laboratory is very useful for medical institutions to decide on treatment and initial investigation policies.

On the other hand, as an organization in charge of analysis, the National Research Institute of Police Science experienced a great deal of confusion in the inspection system during the 1998 poisoning-related incidents. In particular, a) unusual drugs (arsenite, azide, etc.) were used in the cases, and it was difficult to quickly identify the substance causing the poisoning; b) there were misjudgments about the detection of poisonous substances in the early stages of the cases (such as the false detection of cyanide ion in the Wakayama poisoned curry case); and c) there were many copycat crimes in which common commodities like liquid soap were added to food, and the number of forensic appraisals temporarily exceeded the processing capacities of the forensic science laboratories. In light of these past incidents, the police have been trying to establish a systematic toxicological examination system that can detect poisoning-causing substances quickly and accurately in order to respond appropriately to cases of poisoning with unknown causes. In this section, the author discusses the problems of the existing toxicological examination system from the viewpoint of consequence, and he describes the direction of development of analytical technology for accurate and rapid identification of substances causing poisoning with the aim of realizing a safe and secure society. In the case of chemical terrorism such as a sarin gas attack, exposure to the vaporized chemical warfare agent causes immediate poisoning. Therefore, in the immediate aftermath of an incident, it is more important for the first responders to detect at the scene rather than to transport the samples to a specialized analytical laboratory for instrumental analysis. The development of sensors and other measurement technologies can make great contributions to on-site detection. More information on the post-event response to chemical terrorism is provided in Section 1-2, “On-site detection for chemical warfare agents”.

1-1-1-2. Poison chain incidents

1-1-1-2-1. Wakayama poisoned curry case

In forensic science laboratories prior to the 1998 poison chain incidents, based on the past expert experiences, simple tests such as visual inspections of evidence samples, liquidity tests such as pH, pre-tests for cyanide ion, and pre-tests for coloration of the herbicide paraquat were prioritized[3]. This was because hydrogen cyanide was the best known to potential criminals and paraquat was common and easily available at that time. Since hydrogen cyanide is also produced during fires and can cause poisoning, the determination of cyanide in blood samples has been conducted in burned death cases. The pyridine-pyrazolone method is still widely used for the determination of cyanide ions in blood.[9] Although this method is relatively complicated, requiring about 3 hours of operation, it has high specificity for cyanide ion and detection sensitivity of several tens of ng/mL. The pre-tests are followed by organic solvent extraction of the samples, and the organic phase is subjected to GC–MS to screen for non-polar pesticides and drugs. The solution samples are evaporated to dryness and subjected to Fourier transform infrared spectroscopy (FT-IR) analysis for qualitative testing of the major organic constituents in them. In addition, electron probe X-ray microanalysis (EPMA) is performed to detect toxic metals contained in samples. In recent years, the widespread use of LC–MS and the construction of data libraries have made it possible to detect polar substances, including metabolites and degradation products of medicines and toxicants. Furthermore, X-ray diffraction (XRD) equipment enables qualitative testing of crystalline substances.

Three years after the sarin gas incidents of 1994-1995, the Wakayama curry incident occurred on July 25, 1998, in which a number of persons who ate curry and rice at the summer festival site of a local community association in the Sonobe district of Wakayama City immediately complained of nausea, diarrhea, and abdominal pain. The health center initially announced that it was food poisoning, but the symptoms occurred so quickly that questions were raised, and the health center retracted the first report. As the cause of the illnesses was therefore unknown and poisoning could be suspected, the Wakayama Prefectural Police launched an investigation. A positive cyanide reaction was obtained by the pyridine-pyrazolone method for the leftover curry evidence samples sent to the prefectural forensic science laboratory. Four persons died on July 26 and 63 others were taken ill. On July 27, the Wakayama Prefectural Police Headquarters announced the detection of cyanide, and the investigation and treatment proceeded along the lines of administering a cyanide antidote to the patients and clarifying the cyanide distribution route. The symptoms were nausea, diarrhea, and abdominal pain, and a day passed before the deaths, so there were some who doubted this line of thinking.

When the prefectural forensic science laboratory could not detect cyanide in the evidence curry samples again, the National Research Institute of Police Science was commissioned to conduct a more sensitive analysis. However, no cyanide was detected using the HS–GC based cyanide detection method (LOD: several ng/mL) that had been developed by the institute.[1,3,10] Although the possibility of cyanide dissipation and decomposition in the curry samples was suspected, the fact that no toxic concentration of cyanide was detected in the victim's blood sample was judged to be conclusive, and it was presumed that the forensic cyanide detection was a false positive. However, the institute workers failed to find any other poisonous substances in the samples. At that time, a researcher who was working at the institute happened to receive a call about the loss of cyanide gold, and if cyanide gold were the substance that caused the poisoning, it could explain the strong toxicity and the detection of cyanide by the prefectural forensic science laboratory. Since the detection sensitivity of the X-ray fluorescence spectrometry system (several tens of µg/mL) was not very high, the analysis was conducted using a new ion chromatography-mass spectrometry (IC–MS) system. On August 1, the National Research Institute of Police Science reported the detection of arsenic in an evidence curry sample, and on August 4, the cause of poisoning was attributed to arsenite. IC–MS and inductively coupled plasma mass spectrometry (ICP–MS) are not only capable of analyzing arsenic in different forms (oxidation degree), but also the *m/z* of the detected ion As. The advantage of IC–MS and ICP–MS is that the *m/z* 75 of the targeted As ion can be detected without interference from the aggregated ions of plasma gas Ar and chlorine Cl (*m/z* 75).

1-1-1-2-2. Niigata poisoned tea incident

Following the poisoned curry case in Wakayama, 13 employees of a factory office in Ogishima-cho, Niigata City, drank tea at around 8:00 a.m. on August 10 of the same year, after which they complained of numbness in their hands and feet, dizziness, and nausea. Although cyanide and arsenic acid were considered, both the medical institution and the forensic science laboratory of the Niigata Prefectural Police reported the detection of cyanide in an emergency preliminary test before noon. The police, who were cautious about cyanide detection after the false positive result in the Wakayama poisoned curry case, immediately sent evidence samples to the National Research Institute of Police Science, but cyanide was not detected in the stomach contents. The prefectural forensic science laboratory continued the toxicological tests, but no specific poison was detected. EPMA did not reveal any characteristic metals other than Mg in the dried solids of the leftover tea and the pot of hot water in the evidence samples. The prefectural forensic science laboratory observed the specific absorption of azide in the dried solids by FT-IR and confirmed the match with the diffraction image of sodium azide crystal by XRD, and the detection of sodium azide was reported on August 11. However, if sodium azide had been added to a complex matrix sample such as curry, it would have been impossible or very difficult to detect the poison. Currently, the pentafluorobenzyl-derivatized–GC–MS method enables highly sensitive detection and quantification of azide regardless of the sample matrix.[11] During August of 1998, there were several cases of contamination of food and beverages with paraquat, diquat, terebinth oil, dichlorobenzene, cresol, and tetramethrin throughout Japan.[12]

1-1-1-2-3. Nagano cyanide oolong tea incident

On September 1 of the same year, the manager of a supermarket in Suzaka City, Nagano Prefecture, took a PET bottle of oolong tea from a display stand and sipped some of it; he noticed that the tea gave off a strange odor and bitter taste. The prefectural forensic science laboratory detected cyanide ions in the tea. A woman in the neighboring city of Obuse, who heard about the foreign substance contamination case, called the police, and it turned out that her husband, who died early in the morning of August 31 after being diagnosed with acute heart disease, had died after drinking oolong tea purchased at that same supermarket. The victim had already been cremated, but his blood and the remainder of his tea drink had been preserved, and the results of cyanide ion tests on the evidence samples at the National Research Institute of Police Science and the prefectural forensic science laboratory showed that the victim's blood and drink samples had high concentrations of cyanide ions, several µg/mL and about 1 mg/mL, respectively.

The cyanide-containing oolong tea bottle had a hole in the bottom and that hole was sealed with adhesive. It was presumed that the perpetrator drilled a hole in the bottom of the unopened oolong tea bottle, added cyanide ions, sealed the hole with glue, and returned the bottle to the store display stand. Immediately after this incident, many people who purchased bottled drinks checked the bottom of the container.

About 40 copycat incidents of contamination of food and beverages with foreign substances occurred nationwide until they stopped at the end of 1998. The majority involved the contamination of food and beverages with foreign, but common substances found in daily life, and most of them were deranged acts or self-made contaminations without wide consequences. Pesticides and bleaching agents were the main contaminants, but sodium azide was used in Mie, Aichi, and Kyoto prefectures, causing multiple victims. Cadmium was also used in Kyoto. Suspicious food and beverage samples were brought into various forensic science laboratories day and night, and a large number of urgent toxicological analyses were conducted.

1-1-1-3. Toxicological inspection system

1-1-1-3-1. Toward the establishment of an inspection system

In order to solve the problems described in 1-1-1-1 that are associated with poisoning incidents, three matters must be attended to from the viewpoint of analytical chemistry. First, it is necessary to establish analytical methods for poisonous and deleterious substances that have been impossible previously to test for by introducing new analytical technologies, such as the use of high-performance analytical instruments. Second, it is necessary to consider three phases of chemical change: phase I, from the time the toxicant is ingested or added to the time the evidence samples is collected; phase II, during transport and storage of the evidence samples; and phase III, during processing or analysis in the laboratory. Considering that many chemical changes occur, the possible causes of misjudgments should be clarified analytically, and accurate analytical methods that do not produce artifacts should be developed based on an accurate understanding of the change processes of toxic substances in biological or environmental samples. Third, it is necessary to establish a rapid and high-throughput simple test method for screening to select samples for more detailed analyses.

Poisoning cases can be divided into two categories: large scale cases with a large number of victims and cases involving foreign substances in food and drink with no clear victims. Evidence samples can be divided into "field samples" such as leftover food and drink that the police obtain at the scene and "biological samples" of the victims. The latter are organs and blood samples collected during administrative autopsies at university forensic medicine departments in cases of death, and they may be sent to a forensic science laboratory. When a victim is transported to a medical institution such as an emergency medical center, biological samples such as blood and stomach contents are subjected to urgent simple toxicological testing at the hospital laboratory, and in some cases, the police may retain these biological samples and send them to the responsible forensic science laboratory. Biological samples can be categorized as stomach contents (outside the body), where food and drink have not yet been fully absorbed, and blood and organs (inside the body). In large-scale poisoning cases, it is not difficult to detect poisoning-causing substances in the field samples, unless the selected test method is wrong, because the information on the victims’ poisoning symptoms may be available to some extent. In this case, the analyses of biological samples are supplementary for proving poisoning and confirming the course of treatment. In cases where field samples cannot be obtained, it is necessary to identify the substance causing the poisoning from biological samples, which is technically more difficult. In the case of contamination of food and drink with foreign substances, it is not always the case that a poisonous amount is mixed in, for example, no poisonous substance is detected at all, and contaminated ingredients may be detected from outside that were introduced during storage, transportation, and handling of evidence samples.

The evidence samples can be divided into three types: field samples, stomach contents, and blood. Field samples are unspecified in terms of sample matrix, blood is constant, and stomach contents are in between. The concentration of toxins in the samples is lower in the order of field samples, stomach contents, and blood. For field samples, decomposition and disappearance of toxicants can occur, for stomach contents, toxicant stability under acidic conditions is an issue, and for blood, metabolism and postmortem changes must be considered. Therefore, toxicological examination of field samples is advantageous in identifying the poisoning-causing substances. Cyanide poisoning can be taken as an example: if a lethal dose of potassium cyanide is mixed in 200 mL of coffee, the concentration is more than 0.5 mg/mL, and if the coffee is drunk, the cyanide concentration in the stomach contents will be about 0.1 mg/mL. The lethal concentration of cyanide ion in blood is about 3 μg/mL. Detection and quantification of cyanide ion from field samples and stomach contents are relatively easy, but detection from blood requires a highly sensitive analytical method.

1-1-1-3-2. Pre-test methods

In addition to the conventional Schönbein method (for cyanide ion) and the Sep-Pak extraction-dithionite reduction colorimetric method (for paraquat), the iron oxide colorimetric method for azide, which is a poison that attracted attention in some of the poison chain incidents, and the Reinsch method for arsenic have been confirmed to be useful and are now being used as preliminary test methods.[13] In addition, a preliminary test method that can detect cyanide and azide ions within one hour was developed by employing microdiffusion extraction under heating and coloration under heating.[14] The capillary electrophoresis (CE) method[15] and the anion exchange IC method are used for simultaneous detection of toxic anionic compounds by analytical instruments. However, while these methods are suitable for toxicological testing of field samples and stomach contents, they are not sensitive enough for detection in biological samples.

1-1-1-3-3. Conventional measurement techniques

X-ray fluorescence spectrometry is conventionally used in forensic science for the detection of metals, and it is characterized by its ability for rapid measurements in any sample matrix. The sensitivity is around several tens of μg/mL, but improvements in sensitivity have been made by using small area X-ray systems and devising sample adjustments.

FT-IR, EPMA, and XRD were effective in detecting sodium azide in the Niigata poisoned tea case, and they can be used not only for micro object analysis in forensic chemistry, but also in the appraisal of toxicological cases. ICP–MS has a capability for high-sensitivity analysis of toxic metals, and it is useful for testing evidence samples that contain only trace amounts of toxic metals, such as biological samples.

IC instruments are used for water quality analysis, and the suppressor type instruments have high sensitivity (ng/mL level). These IC instrumental analyses are applicable to the examination of field samples and stomach contents.

The head-space method is a pretreatment method for GC analysis and it is suitable for the analysis of volatile substances.[10] Because of its high sensitivity, the head-space method can be applied to all types of evidence samples in any sample matrix. It can detect organic solvents better than GC–MS analysis using a polar column and MS detector. Cyanide and nitrile compounds can be detected by GC analysis using a nitrogen-phosphorus detector (a type of flame photometric detector).[16]

1-1-1-3-4. Derivatization for GC–MS method analysis

The GC–MS method is used for the analysis of non-polar pesticides and drugs that volatilize when heated, but it can also be used for polar compounds that were previously unsuitable for analysis by converting them to volatile compounds through derivatization reactions. Anionic toxicants have been analyzed by IC, but GC–MS analysis is now possible by derivatization with pentafluorobenzyl (PFB).[11]11 Derivatization for a GC–MS method analysis (derivatized–GC–MS method) is a highly sensitive approach and can be applied to almost all types of evidence samples, regardless of the sample matrix.

1-1-1-3-5. New measurement technologies

LC–MS has recently become popular, but in 1998, its use was not widespread in forensic science. LC–MS is essential for the analysis of organic toxicants with molecular weights of several hundred or more, such as natural poisons, unstable medicinal toxicants, and their metabolites and conjugates.

CE is characterized by high resolution, high reproducibility, rapidity, and simplicity,[17] and has been used as a screening method for toxic anions. In addition, it is possible to separate and detect toxic anions, cations, neutral molecules, non-polar molecules, and polar molecules using only one capillary fused silica column by adjusting the phoresis conditions such as the phoresis buffer. CE has also been proposed for use as an adjunct to the analysis of toxic substances themselves in forensic chemical analysis. For example, in the case of cyanide contamination of beverages, the determination of cyanide is absolutely necessary due to the lethal dose, but since cyanide volatilizes and disappears under neutral and acidic conditions, the concentration of cyanide in potential evidence samples decreases with time before they are collected. Since the counter cation of cyanide, e.g., potassium ion, does not volatilize and disappear, its concentration can be quantified by analyzing it in a swimming buffer system containing hydroxyisobutyric acid and 18-crown-6-ether to determine the amount of potassium cyanide added. Since alkali metals and alkaline earth metals are contained in beverages, it is necessary to evaluate the target beverage and subtract the background value. Absolute quantification is possible with the CE method by selecting the phoresis conditions that minimize the influence of the sample matrix. The lower detection limit is not as high as the µg/mL level, but it is sufficient for rapid testing of field samples.

1-1-2. Chemical warfare agents

1-1-2-1. Properties of chemical warfare agents

Most chemical warfare agents are low-molecular-weight synthetic compounds that are effective for suppression (suppression of insurgents, paralysis of enemy forces) at low exposure concentrations.[18, 19, 20] According to their toxic effects and physicochemical properties, these compounds are classified as: gaseous "blood agents" and "choking agents"; volatile "nerve agents" and "blister agents"; and non-volatile "vomiting agents (sneezing agents)" and "tearing agents (mob suppressants)". Molecular weight, melting point, boiling point (vapor pressure), atmospheric retention, lethal concentration, disabling concentration, odor, aqueous solubility, stability in water, skin effects, and antidote efficacy vary greatly from agent to agent. Although there are some slow-acting chemical warfare agents such as phosgene and mustard that become toxic after a long exposure time, onset of action is generally rapid. Nerve agents, which are highly poisonous organophosphorous compounds, and blister agents, which are chlorine-containing compounds, are designated as scheduled chemicals by the Chemical Weapons Convention.[21] In Japan, some chemical warfare agents are treated in accordance with the Chemical Weapons Convention as the Law Concerning the Prohibition of Chemical Weapons and the Regulation of Specified Chemical Substances[22] (effective June 7, 2020). Toxic substances used as chemical warfare agents and their highly-likely-to-be-used raw materials are defined as "specified substances”; other toxic substances and their raw materials that may be used in the manufacture of chemical weapons are defined as "designated substances". The manufacture, possession, and use of both specified and designated substances are legally restricted.

Although chemical warfare agents can be taken up through skin contact or oral ingestion, vapor inhalation is the primary route of ingestion in chemical terrorism and accidents, and major on-site detection technologies target chemical warfare agents in vapor form. Lethal loading values (LCt_{50}, $mg{\cdot}min/m^3$) are used as the values to determine toxicity.[23]

There are four main ways in which chemical warfare agents may be released at terrorist sites: 1) artillery shells are fired and land on the site and explode, scattering chemical warfare agents; 2) chemical warfare agents are dispersed as aerosols by sprayers; 3) chemical warfare agents are sprayed and spread by natural evaporation; and 4) chemical warfare agents are added to food and drink. These forms of chemical warfare agent vapors or aerosols can be inhaled through the respiratory tract, absorbed through the skin, or ingested through the digestive tract, resulting in poisoning. Since inhalation of a chemical warfare agent diffused over a wide area can lead to large-scale poisoning, the volatilization (mg/m^3) and half lethal concentration (LCt_{50}, $mg{\cdot}min/m^3$) are important. Figure 1-3 shows the chemical structures of major chemical warfare agents, and lists their LCt_{50} values, and volatilization (mg/m^3) at 20°C.

The volatilization and LCt_{50} values for sarin are 23,000 mg/m^3 and 150 $mg{\cdot}min/m^3$, respectively. Sarin, which has a volatility higher than its lethal concentration, caused deaths and thousands of injuries to people in the Tokyo subway sarin gas attack that showed how dangerous exposure to it is for unprotected humans. On the other hand, VX and Novichok, which are less volatile and more toxic, are often used for assassination purposes targeting individuals.

Chemical warfare agents that act by inhibiting the cholinesterase (ChE) activity of nerve synapses, causing accumulation of acetylcholine and inhibiting neurotransmission include sarin, VX, and Novichok. Blister agents that act on the skin, eyes and respiratory organs, causing erosion of their

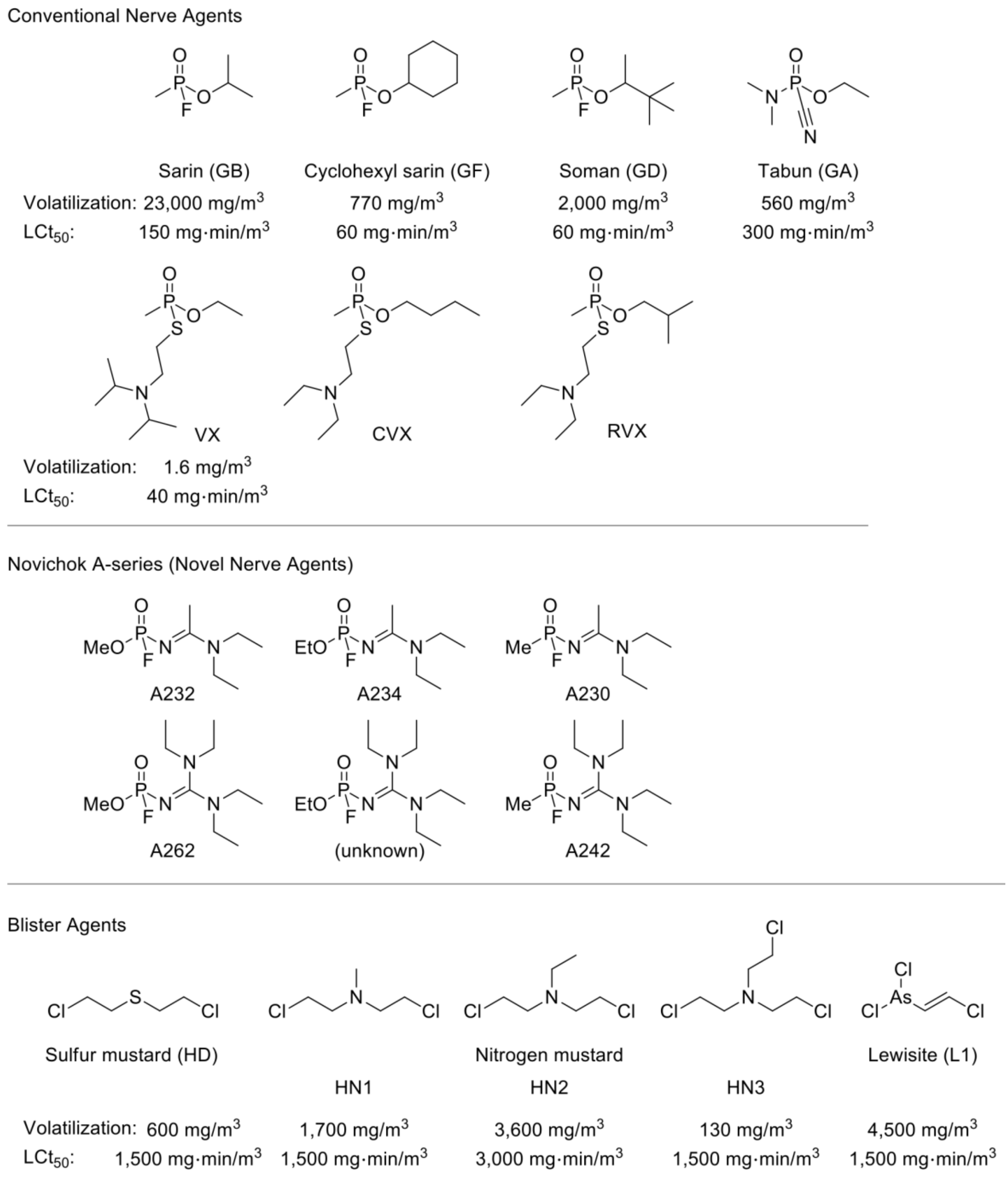


Figure 1-3a. Structure, volatilization, and LCt$_{50}$ of major chemical warfare agents

contact surfaces include mustard gas, nitrogen mustard, and Lewisite 1. Phosgene is an example of choking agents that injure the respiratory system, including the nose, throat, and lungs, making breathing difficult. Hydrogen cyanide is an example of blood agents that interfere with the normal oxygen consumption of cells. 2-Chloroacetophenone is an example of vomiting agents that irritate

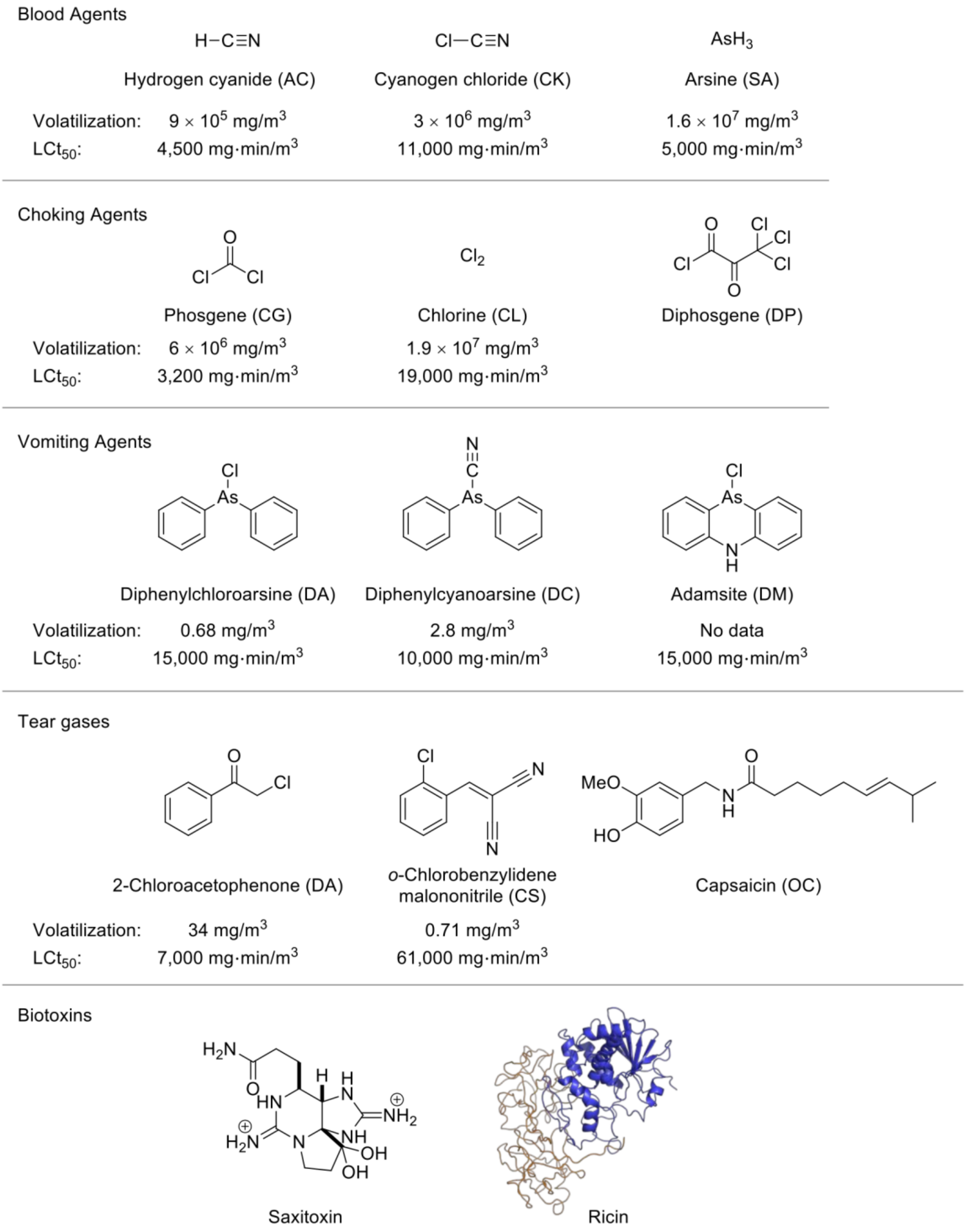


Figure 1-3. Structure, volatilization, and LCt_{50} of major chemical warfare agents

the mucous membranes of the eyes, nose, and pharynx, causing tearing, sneezing, and coughing. Finally, 3-quinuclidine benzilate is an example of tearing agents that temporarily render persons physically or mentally inactive. Except for capsaicin and biotoxins (Figure 1-3), all the above are synthetic chemicals. The nerve agents, blister agents and sneezing agents have only chemical warfare applications, while blood agents and choking agents have industrial applications, as well.

1-1-2-2. Cases in which chemical warfare agents were used

The Iran-Iraq War in the 1980s is a well-known case in which the chemical warfare agents, sarin gas and sulfur mustard, were used.[24] In 1992, the United Nations adopted the Chemical Weapons Convention[25] and in 1994, the Japanese government enacted, the Law Concerning the Prohibition of Chemical Weapons and the Control of Certain Chemical Substances.[22] In 2013, the Organization for the Prohibition of Chemical Weapons (OPCW) was awarded the Nobel Peace Prize.

In Japan, members of the Aum Shinrikyo religious cult manufactured sarin gas in the early 1990s, and released it in incidents in Matsumoto in 1994 and in the Tokyo subway system in 1995, together causing 19 deaths and injuries to more than 5,000 persons.[26,27,28,29,30,31] In addition, the group carried out personal terrorism (using VX) and small-scale terrorism (cyanide gas use was attempted). The Tokyo subway sarin gas attack prompted other countries to put efforts into building a system to deal with chemical terrorism. In Japan, after the poisoned curry incident in Wakayama in 1998,[32,33] the criticality accident at Tokai Village in 1999 (this was not a terrorist attack but an industrial accident that caused the release of radioactive materials, contaminating areas outside the plant), and the 2001 9/11 terrorist attacks and the sending of anthrax-contaminated objects in the mail, the government begun to develop a system to deal with weapons of mass destruction: that is, chemical warfare agents, biological agents, explosives, and radiological and nuclear materials(CBERN).[2]

In the event of a terrorist attack using chemical warfare agents (chemical terrorism), the most important crisis management measure is to detect and identify the causative agent. As indicated earlier in Figure 1-1, achieving this means 1) carrying out monitoring and security checks of hazardous materials at large-scale events, important facilities, etc., to prevent terrorist attacks before they occur (pre-terrorism management); 2) carrying out on-site detection by police and other special teams; and 3) implementing laboratory analysis by transporting on-site samples to specialized analysis laboratories (e.g., toxicological analysis) to deter recurrence from the perspective of proving crimes after a terrorist attack (incident management).

1-2. On-site detection for chemical warfare agents

1-2-1. Overview of on-site detection

In Japan, the administrative departments that deal with chemical weapons are the Ministry of Economy, Trade and Industry (in charge of domestic laws), the Ministry of Foreign Affairs (treaties), the Ministry of Defense (defense), the Cabinet Office (disposal of abandoned chemical weapons in China), and the National Police Agency, the Fire and Disaster Management Agency, and the Japan Coast Guard (counter-terrorism). From the perspective of on-site detection of chemical warfare agents, there are various activities: in the case of disposal of abandoned chemical weapons, monitoring of limited chemical warfare agents at excavation and disposal sites is undertaken; in the case of defense, detection of chemical warfare agents that are expected to be present on the battlefield to some extent is done;[34] and in the case of counter-terrorism, detection of suspicious substances at the border, important facilities, and event sites is done.[35] Although this thesis is limited to chemical terrorism countermeasures, the technology is also common to the disposal of abandoned chemical weapons and defense. Except for obvious cases, such as a statement of intent, the possibility of dispersal of various types of chemical warfare agents, including toxic industrial chemicals (TICs), must be considered. If the symptoms of a victim exposed to a chemical warfare agent are clearly visible, it is possible to narrow down the agent to be detected. Although the rapid onset of toxicity of chemical warfare agents makes it difficult to rescue victims exposed to high concentrations, rapid on-site detection is of paramount importance to prevent the spread of exposure damage, minimize the scale of injuries to victims and damage, and provide effective medical treatment if victims are still alive. However, the performance requirements for on-site detection technology have not been clearly defined, and they are based solely on experience. Hence, they lack sufficient consideration of how first responders can detect chemical warfare agents in the field. The requirements for on-site detection include the types of chemical warfare agent for which an alarm can be made, alarm output, response time, start-up time, recovery time, robustness, alarm portability, operability, maintenance requirements, and installation and maintenance costs. In addition, the working conditions and performance sustainability should also be considered.

There is an opinion that it is important to issue an alarm within one minute for vapor concentrations below 1/100 of the 50% lethal concentration (LC_{50}), based on the diversity of toxicity statement times and dispersal situations in terrorism incidents.[1,36,37,38,39] In the case of sarin, the required detection concentration is 1.5 mg/m^3, and at this level humans show few symptoms and cannot smell the gas. In chemical weapons processing, a time-weighted average (TWA) value (roughly 1/100,000 of the lethal concentration) is an acceptable monitoring indicator in a working environment because workers remain on site for long periods of time.[23] There is a trade-off between alarm sensitivity, alarm time, alarm accuracy, and operability; for example, increasing the alarm sensitivity of a given detector results in longer alarm times and higher false positive rates.

When the initial response team arrives at the scene, the members first assess the damage and the symptoms of the injured, but these are empirical, relying on their senses. At the time of the sarin gas incident, field samples and victim samples were transported to specialized laboratories, and chemical warfare agents were identified through laboratory analysis. In order to minimize the effects of incidents, the ability to obtain information on the type and concentration of chemical warfare agents dispersed as soon as possible is crucial to the success or failure of the response. Science and technology have advanced significantly since 1995, and it is now possible to prove the use, poisoning, and exposure of chemical warfare agents quickly and accurately.[1,2,3,9,40,41,42,43] In terms of facilities, a safe handling environment for scrubber draughts, decontaminants, etc., and skills and experience in handling chemical warfare agents are required for inspectors. One of the major changes is that field air samples can now be collected and concentrated on Tenax adsorbent and analyzed in the laboratory by thermal desorption GC–MS.[44,45,46] The detection of biomacromolecule-agent adducts, which have a long half-life in vivo, is an effective way to prove exposure to chemical warfare agents.[47] For the serum samples of victims exposed to nerve gas, fluorinated products released by sodium fluoride treatment of ChE adducts are analyzed by GC,[48,49] ChE-albumin adducts of nerve agents, albumin or keratin adducts of mustard gas are enzymatically digested, and fragmented peptides are analyzed by LC–MS/MS.[42]

1-2-2. Classification of on-site detection methods by activity type and purpose

Upon arrival at the scene, first responders may suspect chemical warfare agent dispersion based on visual observations. If abnormal conditions are observed at the scene (large numbers of casualties without external injuries and bleeding, suspicious objects, etc.), chemical terrorism is assumed to have occurred, and the type of chemical warfare agent is estimated from the poisoning symptoms of the victims. In the past, when equipment was scarce, the five senses were used to make decisions. However, odor sensitivity and perception vary greatly from person to person, and past reports indicate that some odors are not from the chemical warfare agent itself, but from impurities in the manufactured chemical weapons. Cyanide gas is considered to have an almond odor, but in reality such a description is not appropriate. For chemical warfare agents, in addition to confirming the damage by sensory perception, chemical components are measured by equipment, and various detection techniques are employed depending on the target compound.[50] The level of alarm identification varies between "screening" (broad classification) and "identification" (substance identification). At present, detector papers, gas detector tubes, and ion mobility spectrometry (IMS), flame photometric (FPD), photoionization detection (PID), surface acoustic wave (SAW) detection, FT–IR, GC, MS, and GC–MS devices are used. In addition, chemical sensors, biosensors, and microfluidic analytical systems (μTAS) are actively being developed. With the exception of advanced discrimination techniques such as chromatography and spectrometry measurements, most detection instruments only give alerts for limited types of chemical warfare agents. Typical agents for which alarms are possible are nerve, blister, choking and blood agents, and TICs. Vomiting agents and tearing agents are in principle undetectable due to their volatility, but they have a low priority for detection due to their low toxicity. Nerve agents are the highest priority targets for detection. Although detection equipment and materials have been developed, manufactured, and sold mainly by defense agencies,[51] there is no guarantee that they can be used in the field without any modification to protect the public.

1-2-3. Classification of on-site detection methods by device type and detection principle

“Remote detection" is a way to detect chemical warfare agents from a remote location using spectroscopic techniques. The techniques are divided into "passive detection", which detects infrared light emitted from the target, and "active detection", which detects secondary light (ultraviolet, visible light, infrared light, etc.) emitted from the target after primary light (laser light, etc.) is emitted from a device installed near the site. If a terrorist attack occurs in a space filled with various interfering substances, it is difficult for remote detection to provide the performance needed for on-site detection.

“Aspiration detection” methods represent another way to detect chemical warfare agents in the air and they are of two types: "continuous detection" (continuous monitoring), in which a fixed or mobile device is used to continuously aspirate air, and "individual point detection", in which a mobile device is brought to the site. Continuous detection is a cycle of collecting air and measuring the concentrated components to raise an alarm. In the case of "collection off-site analysis," the collected samples are transported from the site to a specialized laboratory for analysis. Liquid and solid samples on a sprayed surface are detected by contacting them with a detector or by collecting a portion of the sample from the contaminated surface and setting it on the measurement unit of the device. It is also possible to incorporate a mobile laboratory into a large trailer and visit the site for analysis.

Judgment of dangerous situations in terrorist attacks must be prompt. If a victim who is leaving, or approaching from, a hazardous area is disabled or affected, appropriate first-aid measures, such as securing the airway and administering antidotes, should be taken. The duty of the first responders is to determine the hazardous area where protective masks and protective clothing are needed. Rapid raising of alarms, and automatic and simple operation of devices are the most important items for field detection, and low frequency of false positive alarms is next in importance. Frequent false positive alarms during measurement hinders proper and timely field activities. A variety of detection instruments are used by military and defense agencies,[52] and some of them have been introduced into riot police, fire and rescue services, and the Japan Coast Guard for the purpose of protecting the public.

1-2-4. Current status of on-site detection technology

1-2-4-1. Classical methods

Manual detection devices are still used in the field. Their operating principle is detection of chemical warfare agents by visually judging the color change caused by the reaction between an agent and a reagent. A "detection paper" immediately changes color when it comes into contact with the chemical warfare agent liquid, and the sensitivity is at the μ–GCm^3 level (e.g., Toyobo (Japan) detection paper and the US military M8 and M9 detection papers). In the case of Toyobo detection paper, two types of dyes and a pH indicator are incorporated into cellulose paper, which turns brown or orange on contact with droplets of a G-agent (sarin (GB), soman (GD), tabun (GA), etc.), brown or orange on contact with an H-agent (HD, L1, etc.), and black or dark green on contact with a V-agent (VX, RVX, etc.). The reaction is based on the solubility of the chemical warfare agent and most solvents other than water will give false positives. Dimethyl methylphosphonate (DMMP), acetone, toluene, ethyl acetate, etc. are false positives for G-agents, 2-mercaptoethanol, carbon tetrachloride, aniline, etc. are false positives for H-agents, and diethylamine is a false positive for V-agents. Due to the high possibility of these false positive occurrences, their use in civil defense is not practical.

"Gas detector tubes" show a color change when chemical warfare agent vapors are drawn into them, and the sensitivity is generally at the mg/m^3 level. Reagents that exhibit compound-specific color change are fixed to a silica gel in the tube. At the time of use, the test tube is cut at both ends and a predetermined amount is drawn in. Industrial gas detector tubes (e.g. Kitagawa detector tubes from Komyo Rikagaku Kogyo (Japan) and Gastech detector tubes (Australia)) can be used to detect blood agents and asphyxiants, but for chemical warfare agents, Draeger Safety gas detector tubes should be used.[53] For nerve agents, a "phosphate ester" detector tube is used, with a lower limit of confirmation of about 0.1 mg/m^3, and the operation is complicated. For HD, a "thioether" detector tube is used, with a lower limit of confirmation of about 2 mg/m^3, and HD pseudo-agents with a thioether structure show false positives; for L1, an "arsine and organic arsenic compounds" detector tube is used, with a lower limit of confirmation of about 4 mg/m^3, and organic arsenic is detected separately by fractionation. For nitrogen mustard (HN1, HN2, HN3), an "organochlorine nitrogen compounds" detector tube is used, with a lower limit of confirmation around 10 mg/m^3, and false positives for compounds with tertiary amine structures. For hydrogen cyanide and chlorine cyanide, "hydrogen cyanide" and "chlorine cyanide" detector tubes are used, with lower limits of confirmation around 0.3 mg/m^3 and 0.8 mg/m^3, respectively. For phosgene and chlorine, "phosgene" and "chlorine" detector tubes are used, with lower limits of 1 mg/m^3 and 0.8 mg/m^3, respectively. Depending on the type of gas detector tube, the operation can be complicated, involving the breaking of the inner tube and the sequential transfer of the reaction solution. Some detector tubes require several minutes of precise operation in several steps while wearing heavy protective clothing and gloves and with limited visibility.

1-2-4-2. Optical detection methods

FPD devices measure the atomic emission of phosphorus or sulfur produced by combustion with hydrogen gas. The AP2C/AP4C from Proengine (France)[54] is a 500 mL plastic bottle sized automatic detector that can detect vapor and droplets, and can be set to different modes for each element to provide immediate alarms for phosphorus, sulfur, and arsenic. The lower alarm limit is about sub-mg/m^3. FPD devices issue false positives for chemical warfare agents (DMMP, 2-mercaptoethanol, 2-chloroethyl ethyl sulfide, 1,4-thioxane, etc.) and cannot detect compounds that do not contain phosphorus or sulfur atoms. The most used agents in chemical warfare (GB, VX, HD, L1) contain phosphorus, sulfur, or arsenic, making FPD devices effective for defensive purposes, but not suitable for screening for counter-terrorism purposes, as further expansion to detect other elements is difficult in principle.

FID devices measure the ions produced by combustion with hydrogen gas, and have a lower alarm limit of about sub-mg/m^3.[55] Most chemical warfare agents are flammable and can be sensitively but non-specifically detected

PID devices are non-specific detectors that measure the amount of ionization generated by chemical warfare agents as they are ionized by ultraviolet light. The lower alarm limit for the ppbRAE 3000+[56] made by Honeywell (USA) is about several mg/m^3. It is highly sensitive to aromatic compounds, which are easily excited by UV light, but not highly sensitive or specific to chemical warfare agents.

SAW-based chemical sensor technology[57,58] can be used for on-site detection of chemical warfare agents. When a chemical warfare agent is reversibly adsorbed onto a special liquid phase polymer layer, the wavenumber of the SAW changes based on the mass increase. Portable array SAW detectors are commercially available, and the ChemSentry, made by BAE Systems (UK), employs ten different polymers to distinguish between nerve agents, blister agents, and blood agents, and generate an alarm. However, the lower alarm limit is high, the response and recovery time is long, and the frequency of false positives is high.[59]

1-2-4-3. Ion mobility spectrometry

IMS detectors[60,61] are the most commonly used instruments in military operations and public defense against explosives and illegal drugs, as well as chemical warfare agents.[62,63] Air components are ionized at atmospheric pressure by beta rays or a corona discharge, and these reactant ions ionize the chemical warfare agent in the vapor. The group of ions derived from the chemical warfare agent is sent to the drift region and is detected at the end of the drift region. The ion mobility is affected by the charge and the size and shape of the molecules. Spectra are observed at intervals of several to several tens of milliseconds. The chemical warfare agent is identified by the ion mobility of the detected peak and semi-quantified by the peak height. Ammonia and organic solvents can be introduced into the drift region as dopants, improving detection sensitivity and discrimination.[64] Nerve gases, which are easily ionized, are detected with high sensitivity, while blood agents and choking agents that do not form characteristic ions and are in close proximity to reactant ions result in low detection sensitivity. Due to the atmospheric pressure ionization mechanism, IMS instruments are compact and their response is fast. However, the resolution of the target ion peaks is much lower than that of MS instruments, resulting in frequent false positives.

Aspiration type devices use a combination of several IMS cells with different ion mobility and polarity to identify chemical warfare agents by pattern recognition.[65,66] M90 and ChemProX are composite detectors made by Environics Oy (Finland),[67] which use ^{241}Am for ionization; they have 6 and 16 IMS cells, respectively, and an accessory semiconductor cell. For G-agents, a "NERVE" alarm is issued at the lower alarm limit of sub-mg/m^3, and false positives are given for DMMP and other pseudo agents. For HD, L1, and HN3, the lower alarm limit is several mg/m^3, and the alarm is "BLISTER", showing false positives for 2-chloroethyl ethyl sulfide (2-CEES) and other pseudo agents. Responsiveness to blood and choking agents is low.[68] Since the detection sensitivity and specificity of the IMS cells for HD containing sulfur are low, the accessory semiconductor cell is used for detection, but the false positive rate is high. In addition, the detection performance of gaseous chemical warfare agents with low molecular weight is insufficient.[69]

There are handheld IMS-based personal chemical detectors manufactured by Smiths Detection (UK) that consist of a short drift tube for corona discharge ionization and ammonia (supplied in exchangeable packs) as a dopant.[70] The current version, the LCO 3.3, gives alerts by agent name for 10 chemical warfare agents, with a lower limit of sub-mg/m^3 for G-agents and lower limits of around 1 mg/m^3 for HD, L1, and HN3. The lower alarm limit for hydrogen cyanide, chlorine cyanide, phosgene, and chlorine are a few mg/m^3, and the frequency of false alarms increases as the moisture removal filter of the detector degrades.[71]

There are other hand-held IMS detectors offering superior detection sensitivity and accuracy. The SABRE 5000, manufactured by Smiths Detection, can detect nine different chemical warfare agents and provide the name of the agent and an alarm.[72] Its successor, the Multi-Mode Threat Detector (MMTD), can also be used to detect explosive components and drugs. In addition to vapor aspiration, the swab detection mode can also be used to detect less volatile components in wipe samples.

Detectors that combine IMS with several types of sensors are also emerging.[73] The GDA2 from AIRSENS (Germany) combines the IMS technique with a semiconductor sensor, metal oxide sensor, and PID to provide comprehensive detection of chemical warfare agents and TICs.[74] The combined method of solid phase microextraction (SPME) and IMS has also been developed.[75]

1-2-4-4. Vibration spectrometry method

FT-IR systems provide non-destructive identification of chemical warfare agents by measuring their infrared spectra.[76] For air samples, the characteristic absorption peak in the low-wavenumber region is used as an indicator, considering the interference of water and carbon dioxide. Portable devices such as the DX-4000[77] made by Gasmet (Finland) use a multiple reflection method to improve sensitivity. The lower limit of detection is sub-mg/m^3. The VIR-100, 200, and 300 from Japan Spectroscopic Corporation use a sealed structure in the interferometer section, which has the same performance as the interferometers installed in high-performance FT-IR devices, to increase sensitivity.[78] For liquid and solid samples, the HazMatID (Smiths Detection), a portable device that employs an ATR diamond sensor, is used on-site and it has an extensive spectral library for handling suspicious white powder cases.[79]

Portable Raman spectrometers are commercially available. First Defender from Thermo Scientific (USA) has an extensive spectral library and is capable of rapid and easy screening not only for chemical warfare agents but also for explosives, illegal drugs, and suspicious white powders (biological agents).[80] The Progeny ResQ from Rigaku (Japan) can switch between the 785 nm and 1064 nm lasers as excitation light, and is effective for samples containing fluorescent substances.[81]

1-2-4-5. Gas chromatography

GC systems measure the chemical warfare agent peaks that elute in steps from the GC column. The chemical warfare agent is identified by retention time and the response of specific detectors (FID, FPD, etc.). MINICAM (manufactured by O.I. Corporation, USA), an installed type, is capable of highly sensitive detection of nerve agents and blister agents with a detection limit of sub-μg/m^3 within 10 minutes by combining atmospheric collection and thermal desorption systems, and it is used in chemical warfare facilities.[82] GC systems with IMS[83,84] and SAW[85] detectors have also been developed.

1-2-4-6. Mass spectrometry

MS can be used to detect chemical warfare agents in the field by reducing the size and increasing the portability of the MS instruments used in laboratories and adding mechanical shock resistance.[86] Electron ionization allows for the use of extensive mass spectral libraries derived from cleavage ion series. For MS systems used in the field, a hydrophobic membrane inhalation method is used to eliminate oxygen and nitrogen from the ionization region.[87] Chemical warfare agents absorbed by the hydrophobic membrane are introduced into the ionization region, but low molecular weight compounds and strongly adsorbed substances cannot be detected. The portable–MS200 (manufactured by Horiba, Japan), which was developed for TIC measurements in the environment, employs a time-of-flight MS analyzer and pattern recognition data analysis, and can detect GB and HD.[88,89]

Atmospheric pressure chemical ionization (APCI)–MS is a soft ionization method that easily yields molecular-derived ions and it has been applied to field analysis. A field-use MS system has been devised and can be applied to chemical warfare agent field analysis. The direct analysis in real time (DART) technique, in which helium plasma is impinged on the sample surface and the ionized components are analyzed, is being used for chemical warfare agent analysis.[90] Low-temperature plasma can be applied to chemical warfare agent analysis because it non-invasively desorbs and ionizes surface contaminants.[91]

GC–MS technology has also been miniaturized[92] to field specifications and instruments are now commercially available. Laboratory equipment has been converted to a vehicle-mounted system for testing liquid samples (extracted with organic solvents in the field), and online systems for concentrating and thermally desorbing field air with adsorbents are in use.[93] The transition to the portable type was accomplished by adopting low-thermal-mass columns and providing a temperature rise program.[94,95] The Hapsite (manufactured by Inficon, USA) was developed as an instrument dedicated to atmospheric analysis, which adsorbs and collects air with the TenaxTA packed adsorption system, thermally desorbs it, and introduces it into a non-polar capillary column to separate components with a temperature rise program, and then performs electron ionization–MS.[96,97] The latest version, the Hapsite ER,[98] has improved performance[99] by using Tri-Bed collection tubes and thermal desorption probes,[100] and can separate and detect GB, GD, GA, GF, HD, HN1, HN2, HN3, and CN in about 10 minutes. The detection limit is generally sub-mg/m^3 under typical conditions. Some gaseous chemical warfare agents can be detected by directly introducing air into the MS section. However, non-volatile VX is not detectable, and the highly adsorbable L1 and *o*-benzylidene malononitrile are not highly detectable. The Torion T-9,[101] a portable GC–MS analyzer made by PerkinElmer (USA), employs a toroidal ion trap–MS analyzer, and can detect chemical warfare agents after electron ionization–GC separation by SPME-type sample introduction[102,103].

1-2-4-7. Sensors

Chemical sensors have also been used for chemical warfare agent detection.[104,105] Electrochemical sensors such as those manufactured by Riken Keiki[106] enable highly sensitive detection of gaseous chemical warfare agents, and highly sensitive detection of HD has been achieved by using carbon fiber electrodes with dispersed gold nanoparticles.[107] A chemical sensor using fluorescent reagents reactive to nerve agents has been reported also.[108] Since nerve agents and blister agents decompose easily, the capillary electrophoresis µTAS system can be used to detect chemical warfare agent decomposition products in on-site samples.[109]

The development of biosensors that utilize the target enzymes of chemical warfare agents is also being attempted.[110] Acetylcholinesterase (AChE) is immobilized on a sensor chip and used for nerve agent detection,[111] where the rate of acetylcholine (ACh) degradation is reduced by enzyme inhibition, and the choline produced by enzymatic degradation of ACh is oxidized by choline oxidase, and the generated hydrogen peroxide is converted into an electrical signal.[112,113,114] Biosensors that utilize organophosphorus hydrolase have also been reported.[115] A biosensor that detects hydrogen cyanide by electrochemically measuring the decrease in degradation of the substrate polyphenol by hydrogen cyanide, in which tyrosinase is immobilized on a carbon glass membrane and concentrated in a mineral colloid, has also been reported.[116]

1-2-5. Comparison of on-site detection methods

The detection performance requirements desired by first responders using detection equipment in the field include detection sensitivity, detection accuracy, response time, return time, and operability. Remote detection is automatic and continuous, and especially the active type is more sensitive, although the equipment is huge. However, background interference is strong, making it problematic for practical use in public defense applications.

Gas detector tubes are capable of detecting a wide range of chemical warfare agents, but they are cumbersome to operate and do not respond quickly enough, and they are regarded as a supplementary method for confirming chemical warfare agent species. Detection papers are non-specific and cannot be used for counter-terrorism. IMS devices achieve rapid and sensitive detection for nerve gases and blister agents, but have low detection sensitivity for gaseous chemical warfare agents, resulting in frequent false positives. In particular, the disadvantages are more pronounced in aspiration-type devices, which have been eliminated to some extent in the long-drift type. FPD devices achieve rapid and sensitive detection of nerve gas and HD, but false-negative results for chemical warfare agents are frequent. PID devices have no detection specificity, and the adsorption of chemical warfare agents is significant.

FT-IR systems are non-destructive rapid detectors, but they have poor detection sensitivity due to difficulty in eliminating interferences. Atmospheric enrichment–GC–MS systems provide sensitive identification of volatile nerve agents and blister agents, but are cumbersome to operate and slow to respond. The membrane electron ionization–MS system has low sensitivity and has a limited range of detectable agents. The APCI–MS system is capable of sensitive detection of volatile chemical warfare agents, but is large and cumbersome to operate. A variety of sensor methods have been devised, but they are currently only applicable to individual agents, and practical devices have yet to be developed. A counter-current APCI–MS system can provide specific, highly sensitive and immediate detection of most chemical warfare agents, but the system is large and has not yet been commercialized

Chemical warfare agents are distributed over a wide range depending on their volatility and inhalation toxicity. In general, commercial detectors are not very good at detecting gaseous and non-volatile chemicals. In addition, they generally have high false positive rates and slow response times. There are no technologies or devices that meet all the criteria for chemical warfare agent detection, and a combination of multiple devices is required.

1-3. Laboratory analysis of nerve agents

1-3-1. Overview of laboratory analysis of chemical warfare agents

Chemical warfare agents vary in toxicity, strength, onset time, and physical properties, but they are generally unstable and lose their toxicity by degradation in water. Once taken into the body, they are metabolized and degraded, and there is little chance that the intact chemical warfare agent itself will be detected. The analysis of such chemical warfare agents can be broadly divided into verification analysis in chemical inspections related to the Chemical Weapons Convention,[21] forensic analysis related to criminal investigations involving chemical warfare agents, and crisis management analysis related to chemical terrorism.[2] In Japan, the Japan Chemical Evaluation and Research Institute (CERI) has been developing analytical methods and conducting inspections. International recommendations have been proposed for chemical inspections.[117] These tests are mainly aimed at identifying chemical warfare agents and related substances in shells and the environment, and GC–MS is positioned as the standard analytical method. A review of general analytical methods for chemical warfare agents[118] and a review of analytical methods for derivatization of degradation products[119] are available. As for forensic analysis, the *Toxicological Methods and Commentary 2017* provides a general explanation of chemical warfare agent analysis.[9] In addition, there are reviews on analytical methods for nerve agents[120] and analytical methods related to the sarin gas incident.[121,122]

Laboratory analysis for consequence management requires rapid and accurate identification of agents. While the forensic science laboratory at each prefectural police headquarters is responsible for dealing with terrorism, the National Research Institute of Police Science, is commissioned by the prefectural police headquarters to conduct the analysis. In addition to the usual chemical analysis skills and experience, it is advisable to have a safe handling environment (scrubber drafts, dedicated laboratory facilities, detection materials and equipment, antidotes, decontaminants) and to have researchers with skills and experience specific to chemical warfare agents handle the samples. Samples include aqueous solutions of field samples (wastewater, environmental water, decontamination solutions), soil, field air, solids (rubber, polymeric materials, paint, clothing), and wipes (oily deposits, dust, residues). Biological samples include blood, urine, skin, hair, and organs of victims exposed to chemical warfare agents. Chemical warfare agents are present in sufficient quantities to cause human poisoning when dispersed in the field, but they decompose after dispersal, during the time persons are rushing to the site to collect and transport samples, during sample storage, and during analytical operations (especially during pretreatment). With the exception of VX and nitrogen mustard, nerve agents, blister agents, and sneezing agents degrade relatively quickly when dissolved in water,[19] and are rapidly degraded and metabolized in vivo. The degradation of nerve agents is particularly rapid under alkaline conditions. Therefore, in practice, it is necessary to be able to detect and identify their metabolism and degradation products in addition to the chemical warfare agents themselves.[123] Nerve agents are hydrolyzed to alkylmethylphosphonates, which eventually become methylphosphonates.[124] Tabun degrades by a different route (Figure 1-4). It is also

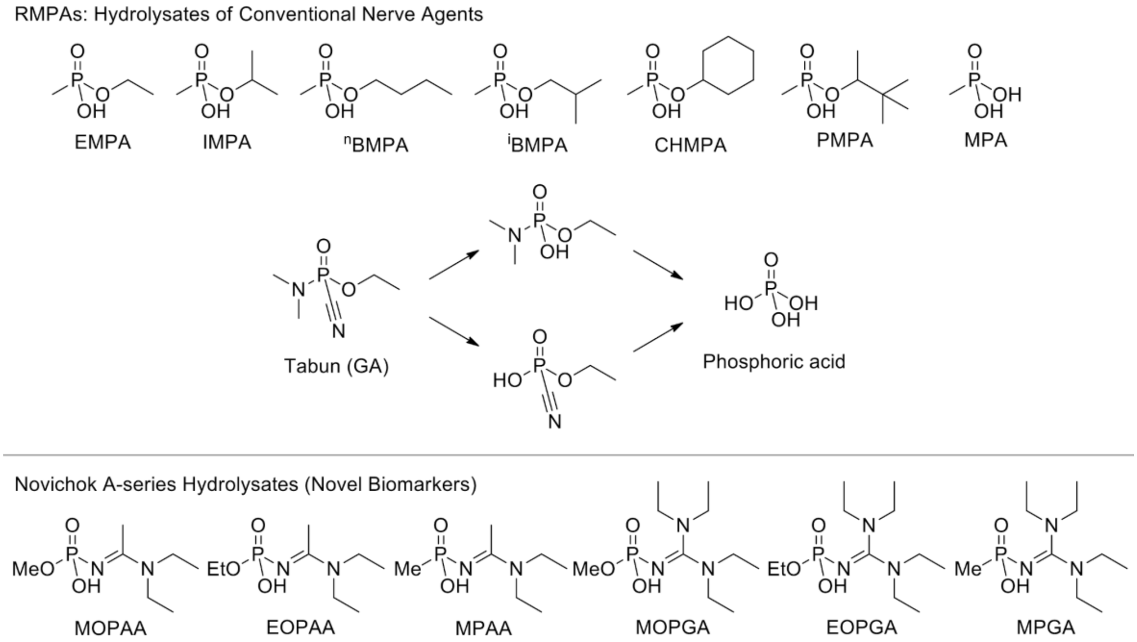


Figure 1-4. Hydrolysis products of nerve agents

metabolized in the body and converted into hydrolysates, oxidized products, conjugates, and adducts with macromolecules such as proteins.

Blood agents and choking agents are low boiling point compounds that scatter easily and are difficult to collect on site, but if air samples can be obtained, they can be qualitatively and quantitatively analyzed by GC systems and gas detection tubes. Cyanide gas can be analyzed by HS–GC method if it is collected in alkaline water on site. Nerve agents, mustard gas, and nitrogen mustard in the air are unlikely to be detected in the laboratory because of their strong adsorption. They can be detected sensitively if adsorbed and concentrated on Tenax in the site, and then injected by GC into a thermal desorption system in the laboratory. Lewisite 1 cannot be directly collected in field air samples, but if it is collected and absorbed in a solution containing dimercapto compounds, the derivatization product can be detected by GC analysis in the laboratory. Non-volatile nerve agents (VX, Novichok), vomiting agents, and tearing agents, which have high boiling points, are impractical to collect from the air and are detected in field wipe samples. As for degradation products, methylphosphonates, thiodiglycols, and ethanolamines characteristic of chemical warfare agents can be detected by LC–MS analysis. The derivatized–GC–MS method and LC-ICP–MS can also be used to detect these compounds.[125]

Although chemical warfare agents are unlikely to be detected directly in the blood of victims exposed to them, cyanide can be easily detected by the HS–GC method, and stable capsaicin can also be detected. On the other hand, for nerve agents, blister agents, and sneezing agents, their metabolites provide evidence of exposure; LC–MS, derivatized–GC–MS, and LC-ICP-MS methods can be used. For mustard gas, detection of characteristic *β*-lyase metabolites is effective.[126]

In addition, biopolymer adducts with a long half-life in the blood are useful as proof of exposure. For nerve agents, ChE and chymotrypsin,[127,128] for blister agents, hemoglobin, albumin, and DNA, and phosgene and albumin adducts are identified by derivatized–GC–MS and LC–MS methods using proteases and chemical reactions.[47] In addition, ChE adducts of nerve agents can be analyzed by GC–MS after the addition of sodium fluoride to regenerate the nerve agent from the adducts to prove exposure.[129,130]

1-3-1-1. Laboratory analysis of nerve agents

The main focus of analysis in the Aum Shinrikyo sarin attacks was to prove the presence of sarin and its hydrolysate in the evidence samples. Since the half-life of sarin in water is about one day and its degradation in the body is rapid, it is effective to detect the hydrolysate as evidence of the presence of the nerve agent. Samples of water and sediment collected at the site are placed in glass vials and covered with Teflon-sealed caps. Any surface exposed to the chemical warfare agent is wiped with a dry cotton swab that is then put into a vial. These field samples are placed in sealed stainless steel containers lined with activated charcoal to prevent secondary exposure during transport and storage, and transported to a forensic science laboratory. Biological samples from autopsies or treatment of the victims are also transported to the forensic science laboratory in vials. If nerve agent poisoning is suspected, blood samples are separated into blood cells and plasma by centrifugation, and ChE activity is measured by DNTB absorption spectrophotometry using acetylthiocholine and butyrylthiocholine as substrates, respectively, to prove nerve agent exposure. In the separated plasma, butyrylcholinesterase is isolated by procaine amide affinity chromatography and SDS-polyacrylamide gel electrophoresis, and the chymotrypsin digestion-LC–MS method was used to detect nerve agent adducts.[128]

Water samples are extracted with dichloromethane at neutral pH. Wipe samples and sediment samples are extracted using dichloromethane, followed by water extraction, and then both are combined and extracted by shaking. For blood samples, after protein removal with perchloric acid, the pH is returned to neutral and extracted with dichloromethane. The organic phase is removed and analyzed by GC. Non-polar compounds such as intact agents, synthetic intermediates, and by-products are detected. In the aqueous phase, hydrolysis products of chemical warfare agents are detected.

Nerve agents and blister agents, which are non-polar chemical warfare agents, are hydrolyzed to highly polar methylphosphonic acids, thiodiglycols, and ethanolamines. Since GC–MS analysis of these degradation products is extremely difficult, derivatized–GC–MS methods have been mainly used for analysis. In recent years, there have been many reports of LC–MS analysis, but still no method has emerged that well meets the desired analytical performance and practicality. In addition, it has been difficult to analyze Novichok degradation products using the conventional

LC–MS method. The details of this situation and the new derivatized–LC–MS/MS method that the author developed to overcome this problem are described in Chapter 3.

1-4. Summary of this chapter

In the case of incidents or terrorism in which poisonous or chemical warfare agents are used, it is important for consequence management to detect and identify the causative agents by laboratory analysis and on-site detection. The results of research on instrumental analysis techniques in toxicology are closely related to the establishment of laboratory analysis methods, and the results of research on sensor-related techniques contribute to the development of on-site detection methods.

Cyanide ions and nerve agents, which are typical poisons and have high toxicity and availability, have attracted the interest of many analytical chemists and sensors, and their analytical methods have been reported. They have also received attention from analytical chemists because of the analytical difficulty derived from their chemical properties. However, even today, while their hazards are fully recognized, practical analytical methods are lacking, especially in terms of simplicity and detection sensitivity.

The situations surrounding the analysis of cyanide ions and nerve agents are summarized in Figures 1-5 and 1-6. The light blue crosses mark areas where it is difficult to conduct sufficient or reliable police work on cyanide ions and nerve agents. In addition, for each of the areas of difficulty, the limitations of the currently available methods are indicated. For cyanide ions, there is no highly sensitive analytical method that can quantify the concentration of the ions to the level of poisoning without producing false positives, regardless of the matrix in the consequence management. In addition, the conventional pretreatment of microdiffusion tends to induce false positives and requires a few hours, which is an obstacle to rapid and simple screening.

As for nerve agents, there has been a serious problem that non-volatile nerve agents (VX, Novichok), which have been used several times in recent years, could not be detected in the on-site detection, which plays a decisive role in the identification of unknown residues in consequence management. In addition, even in the case of incident management, there is no general-purpose analytical method with sufficient detection sensitivity for nerve agent degradation products, and no research on Novichok has been done in this area.

The work in this thesis was aimed at overcoming these deficiencies or shortcomings by using newly found chemical reactions and newly found device designs so as to contribute to the safety and security of citizens.

The following chapters discuss the work in detail.

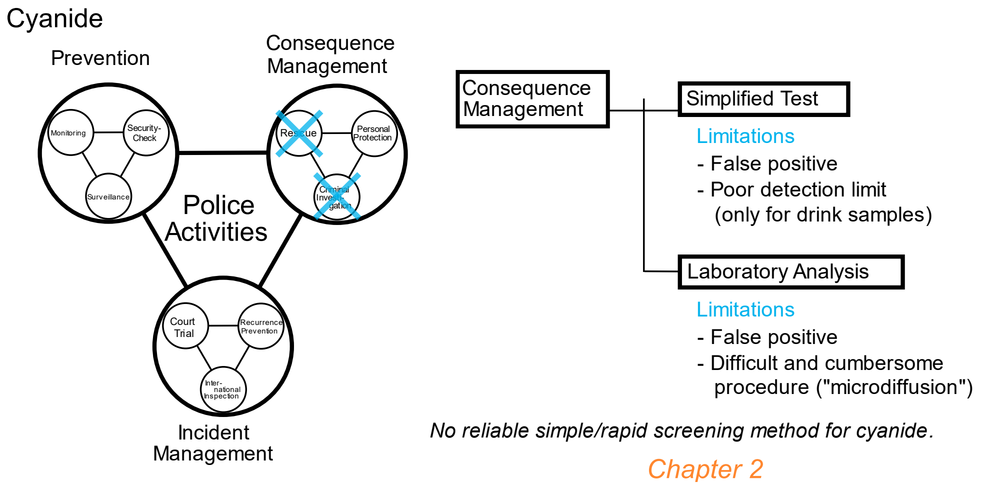


Figure 1-5. Limitations in dealing with cyanide poisoning cases

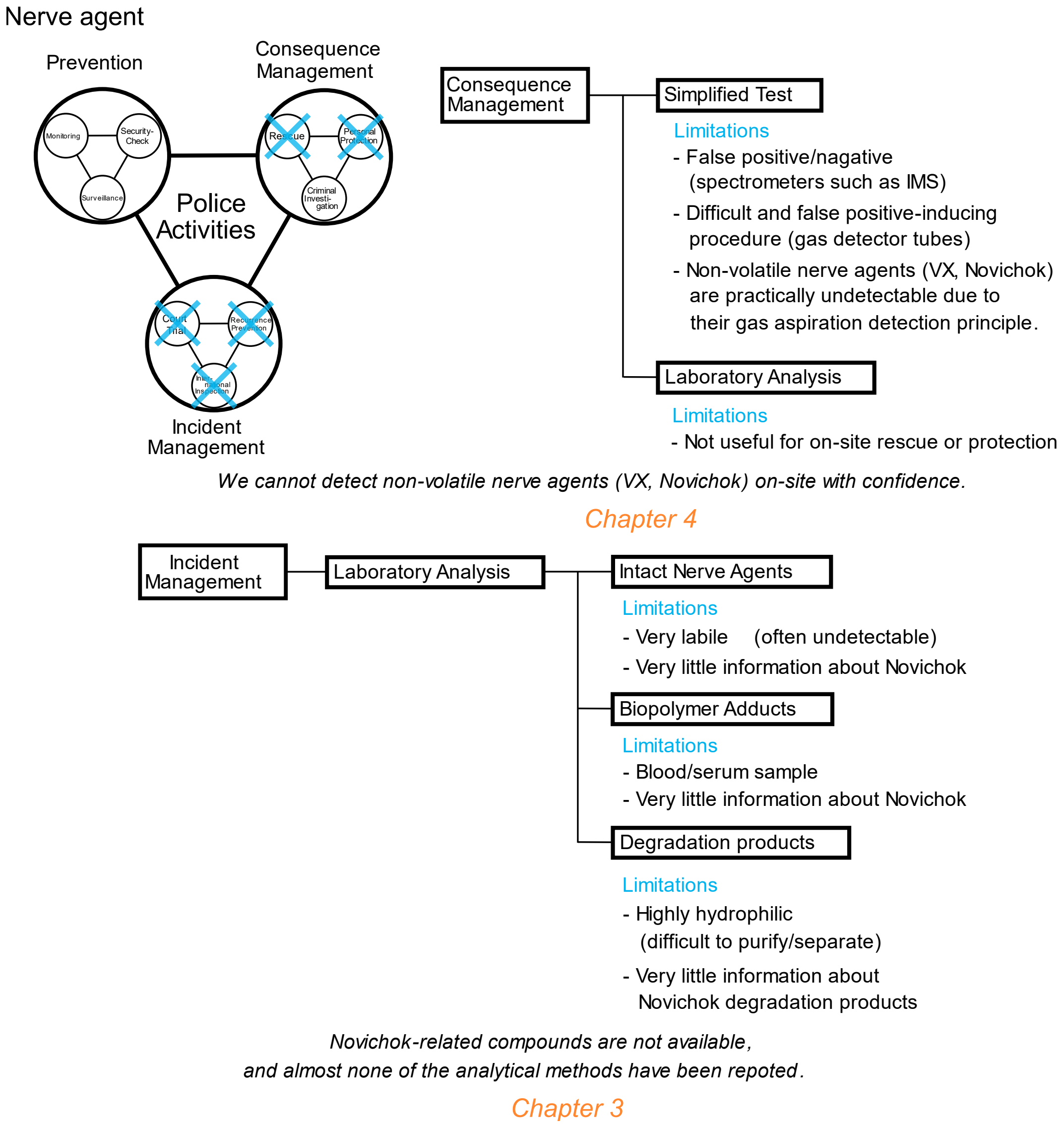


Figure 1-6. Limitations in dealing with nerve agent poisoning cases

1-5. References

---

[1] Seto, Y. Forensic and Analytical Chemistry: A Review. *Bunseki* **2014**, *3*, 19–24.

[2] CBRNE Study Group on Combating Terrorism *The Essential NBC Counterterrorism Handbook*; Shindan to Chiryou-sha: Tokyo, Japan, 2008.

[3] Seto, Y. Analytical and On-site Detection Methods for Chemical Warfare Agents. *Yakugaku Zasshi* **2006**, *126*, 1279–1299.

[4] Reuters, U.N. conforms sarin used in Syria attack; U.S., U.K., France blame Assad. 2013, https://jp.reuters.com/article/uk-syria-crisis-un/u-n-confirms-sarin-used-in-syria-attack-u-s-uk-france-blame-assad-idUKBRE98F10520130916 (accessed 2 December 2021).

[5] Ministry of Health, Labour and Welfare: Promotion of the investigation of the cause of death, https://www.mhlw.go.jp/stf/seisakunitsuite/bunya/kenkou_iryou/iryou/shiinkyuumei.html (accessed 2 December 2021).

[6] Ministry of Health, Labour and Welfare: About the Headquarters for the Promotion of Determination of the Cause of Death and the Study Group, https://www.mhlw.go.jp/stf/seisakunitsuite/bunya/kenkou_iryou/iryou/shiinkyuumei_honbu.html (accessed 2 December 2021).

[7] Nihon Keizai Shimbun: Police Agency to Test All Dead Bodies for Poisons from FY2004 in Response to Serial Cyanide Murders, https://www.nikkei.com/article/DGXLASDG12H8I_S5A111C1CC1000/ (accessed 2 December 2021).

[8] Ministry of Internal Affairs and Communications: Policy Evaluation Report on the Promotion of the Investigation of the Cause of Death (March 2021) https://www.soumu.go.jp/main_content/000737914.pdf (accessed 2 December 2021).

[9] The Pharmaceutical Society of Japan *Yakudokubutsu shikenhou to chukai 2017*; Tokyo Kagaku Dojin: Tokyo, Japan, 2017.

[10] Seto, Y. *J. Chromatogr. A* **1994**, *674*, 25–62.

[11] Tsuge, K.; Seto, Y. *Kanshiki Kagaku* **2002**, *7*, 19–35.

[12] Food Safety Commission of Japan: Investigation of the "Wakayama Poisoned Curry Case”, https://www.fsc.go.jp/fsciis/attachedFile/download?retrievalId=cho20040300015&fileId=02-002 (accessed 2 December 2021).

[13] Department of Forensic Medicine, Hiroshima University School of Medicine *Simple Test Methods for Drugs and Poisons*; Jihou: Tokyo, Japan, 2001.

[14] Tsuge, K.; Kataoka, M.; Seto, Y. *J. Anal. Toxicol.* **2001**, *25*, 228–236.

[15] Soga, T.; Tajima, I.; Heiger, D. N. *Am. Lab.* **2000**, *32*, 124–128.

[16] Shibata, M.; Inoue, K.; Yoshimura, Y.; Nakazawa, H.; Seto, Y. *J. Health Sci.* **2004**, *78*, 301–305.

[17] Seto, Y.; Kanamori, M. *Kanshiki Kagaku* **2003**, *8*, 1–19.

---

[18] Stewart, C. E.; Cullivan, J. B. Jr. *Military munitions and antipersonnel agents. in: Hazardous Materials Toxicology–Chemical Principals of Environmental Health (Sullivan, J. B. Jr, Krieger, G. R. eds.).* pp. 986–1014; Willams and Wilkins, Baltimore, MD, 1992.

[19] Somani, S. M. *Chemical Warfare Agents*; Academic, San Diego, CA, 1992.

[20] Marrs, T. C.; Mayland, R. L.; Sidell, F. R. *Chemical Warfare Agents: Toxicology and Treatment*; John Wiley & Sons, Chichester, UK, 1996.

[21] Organization for the Prohibition of Chemical Weapons: Annex on Chemicals, Chemical Weapons Convention, https://www.opcw.org/chemical-weapons-convention/annexes/annex-chemicals/annex-chemicals (accessed 2 December 2021).

[22] Ministry of Economy, Trade and Industry: Outline of the Law of the Prohibition of Chemical Weapons and the Regulation of Specific Substances, https://www.meti.go.jp/policy/chemical_management/cwc/domestic_law.html (accessed 2 December 2021).

[23] Ellison, D. *Handbook of Chemical and Biological Warfare Agents*; CRC Press, Boca Raton, France, 2000.

[24] Black, R. M.; Clarke, R. J.; Read, R. W.; Reid, M. T. J. Application of gas chromatography–mass spectrometry and gas chromatography–tandem mass spectrometry to the analysis of chemical warfare samples, found to contain residues of the nerve agent sarin, sulphur mustard and their degradation products, *J. Chromatogr. A* **1994**, *662*, 301–321.

[25] Organization for the Prohibition of Chemical Weapons, Chemical Weapon Convention, https://www.opcw.org (accessed 2 December 2021).

[26] National Police Agency, Japan, *Police White Paper*, 1995, 1996.

[27] Nagano, T.; Tsunoda, N.; Seto, Y.; Miyake, B. Indiscriminate terrorism by use of chemical warfare agents. *Adv. Legal. Med.* **1997**, *3*, 284–289.

[28] Seto, Y.; Tsunoda, N.; Kataoka, M.; Tsuge, K.; Nagano, T. *Toxicological analysis of victim's blood and crime scene evidence samples in the sarin gas attack caused by the Aum Shinrikyo cult. in: Natural and Selected Synthetic Toxins-Biological Implications* (Tu, A. T.; Gaffield, W. eds.), pp. 318–332, American Chemical Society, Washington, DC, 2000.

[29] Seto, Y. *Sarin gas attacks in Japan and forensic investigations–A case report–. in: Science, Technology, and National Security* (Majumder, S. K.; Resenfeld, L. M.; Miller, E. W.; Rieders, M. F.; Alexande, S. S.; Panah, A. I. eds.), pp. 7488, Pennsylvania Academy of Science, Easton, PA, 2002.

[30] Seto, Y. *The Sarin gas attacks in Japan and the related forensic investigation in Treaty Enforcement and International Cooperation in Criminal Matters* (Yepes- Enriquez, R.; Tabassi, L. eds.), pp. 301–307, T. M. C. Asser Press, The Hague, 2002.

[31] Okumura, T.; Seto, Y.; Fuse, A. Countermeasures against chemical terrorism in Japan. *Forensic Sci. Int.* **2013**, *227*, 2–6.

---

[32] Seto, Y. False cyanide detection. *Anal. Chem.* **2002**, *74*, 134A–141A.

[33] Seto, Y. Analytical Chemistry Contributing to Crisis Management in Poisoning Cases. *Bunseki Kagaku*, **2007**, *56*, 981–991.

[34] Shidell, F. R.; Patrick, W. C.; Dashiell, T. R. *Detection equipment. Jane's Chem-Bio Handbook, 2nd ed., Ch. 8.1.*, Jane's Information Group, Surrey, UK, 2003.

[35] Harris, C. M. The science of detecting terror. *Anal. Chem.* **2002**, *74*, 126A–133A.

[36] Seto, Y.; Kanamori, M.; Tsuge, K.; Ohsawa, I.; Matsushita, K.; Sekiguchi, H.; Itoi, T.; Iura, K.; Sano, Y.; Yamashiro, S. Sensing technology for chemical-warfare agents and its evaluation using authentic agents. *Sens. Actuat. B.* **2005**, *108*, 193–197.

[37] Seto, Y.; Maruko, H.; Sekiguchi, H.; Sano, Y.; Yamashiro, S.; Matsushita, K.; Itoi, T.; Iura, K.; Kanamori-Kataoka, M.; Tsuge, K.; Ohsawa, I. Development of on-site detection method for chemical and biological warfare agents. *J. Toxicol. Toxin Rev.* **2007**, *26*, 299–312.

[38] Seto Y. *Handbook of the Toxicology of Chemical Warfare Agents, 2nd ed.* (Gupta R. C. eds.), pp. 897–914, Elsevier, Amsterdam, 2015.

[39] Seto, Y. On-site detection as a countermeasure to chemical warfare/terrorism. *Forensic Sci. Rev.* **2014**, *26*, 23–51.

[40] Kanamori-Kataoka M.; Seto Y. Laboratory Identification of the Nerve Gas Hydrolysis Products Alkyl Methylphosphonic Acids and Methylphosphonic Acid, by Gas Chromatography-Mass Spectrometry after tert-Butyldimethylsilylation. *J. Health Sci.* **2008**, *54*, 513–523.

[41] Seto Y.; Kanamori-Kataoka M. Mass Spectrometric Strategy for the Determination of Natural and Synthetic Organic Toxins *J. Health Sci.* **2005**, *51*, 519–525.

[42] Tsuge, K.; Seto Y. Mass Spectrometric Identification of Chemical Warfare Agent Adducts with Biological Macromolecule for Verification of Their Exposure. *J. Health Sci.* **2009**, *55*, 879–886.

[43] Seto Y.; Kanamori-Kataoka M.; Tsuge, K. Mass Spectrometric Technologies for Countering Chemical and Biological Terrorism Incidents *J. Mass Spectrum. Soc. Japan* **2008**, *56*, 91–115.

[44] Degenhardt, C. A. M.; Pleijsier, K.; vander Schans, M. J.; Langenberg, J. P.; Preston, K. E.; Solano, M. I.; Maggio, V. L.; Barr, J. R. Improvements of the fluoride reactivation method for the verification of nerve agent exposure. *J. Anal. Toxicol.* **2004**, *28*, 364–371.

[45] Van der Scans, M. J.; Polhuijs, M.; van Dijk, C.; Degenhardt, C. E. A. M.; Pleijsier, K.; Langenberg, J. P.; Benschop, H. P. Retrospective detection of exposure to nerve agents: analysis of phosphofluoridates originating from fluoride-induced reactivation of phosphylated BuChE. *Arch Toxicol.* **2004**, *78*, 508–524.

[46] Koller, M.; Becker, C.; Thiermann, H.; Worek, F. GC–MS and LC–MS analysis of nerve agents in body fluids: Intra-laboratory verification test using spiked plasma and urine samples. *J. Chromatogr. B* **2010**, *878*, 1226–1233.

[47] Noort, D.; Benschop, H. P.; Black, R. M. Biomonitoring of exposure to chemical warfare agents: a review. *Toxicol. Appl. Pharmacol.* **2002**, *184*, 116–126.

---

[48] Pulhuijs, M.; Jandenberg, J. P.; Benschop, H. P. New method for retrospective detection of exposure of organophosphorous anticholinesterases: application to alleged sarin victims of Japanese terrorists. *Toxicol. Appl. Pharmacol.* **1997**, *146*, 156–161.

[49] Seto Y.; Kanamori-Kataoka M.; Komano, A.; Nagoya, T.; Sasano, R.; Matsuo, S. Gas chromatography-mass spectrometry with spiral large-volume injection for determination of fluoridated phosphonates produced by fluoride-mediated regeneration of nerve agent adduct in human serum. *J. Chromatogr. A* **2019**, *1583*, 108–116.

[50] Paddle, B. M. Biosensors for chemical and biological agents of defense interest. *Biosens. Bioelectron.* **1996**, *11*, 1079–1113.

[51] Smith, W. D. Analytical chemistry at the forefront of homeland defense. *Anal. Chem.* **2002**, *74*, 462A–466A.

[52] Fittch, J. P.; Raber, E.; Imbro, D. R. Technology challenge in responding to biological and chemical attacks in the civilian sector. *Science* **2003**, *302*, 1350–1354.

[53] Takayama, Y.; Sekioka, R.; Sekiguchi, H.; Maruko, H.; Ohmori, T.; Seto, Y. Chemical agent detection performance of gas detector tubes. *Bunseki Kagaku* **2007**, *56*, 355–362.

[54] PROENGIN: AP2C/AP4C, https://www.proengin.com/en/product/15/cbrn-system-accessories/17/ap2c-ap4c-simtoolkit (accessed 2 December 2021).

[55] INFICON: MicroFID II portable flame ionization detector, https://www.inficon.com/v1/attachment/MicroFIDII-Brochure (accessed 2 December 2021).

[56] Honeywell: ppbRAE 3000+, https://sps.honeywell.com/us/en/products/safety/gas-and-flame-detection/portables/ppbrae-3000 (accessed 2 December 2021).

[57] Grate, J. W. Acoustic wave microsensor arrays for vapor sensing. *Chem. Rev.* **2000**, *100*, 2627–2648.

[58] Harris, C. M. Seeing SAW potential. *Anal. Chem.* **2003**, *75*, 355A–358A.

[59] Matsushita, K.; Sekiguchi, Y.; Seto, Y. Performance of Portable Surface Acoustic Wave Sensor Array Detector for Chemical Agents. *Bunseki Kagaku* **2005**, *54*, 83–88.

[60] St. Louis, R. H.; Hill, Jr. H. H. Ion mobility spectrometry in analytical chemistry. *Crit. Rev. Anal. Chem.* **1990**, *21*, 321–355.

[61] Eiceman, G. A.; Karpas, Z. *Ion mobility spectrometry, 2nd ed*, Taylor & Francis, Boca Raton, FL, 2005.

[62] Cottingham, K. Ion mobility spectrometry rediscovered. *Anal. Chem.* **2003**, *75*, 435A–439A.

[63] Eiceman, G. A.; Stone, J. A. Ion mobility spectrometers in national defense. *Anal. Chem.* **2004**, *76*, 390A–397A.

[64] Puton, J.; Nousiainen, M.; Sillanpaa, M. Ion mobility spectrometers with doped gases. *Talanta* **2008**, *76*, 978–987.

[65] Zimmermann, S.; Abel, N.; Baether, W.; Barth, S. An ion-focusing aspiration condenser as an ion mobility spectrometer. *Sens. Actuat. B* **2007**, *125*, 428–434.

---

[66] Zimmermann, S.; Barth, S.; Baether, W. K. M.; Ringer, J. Miniaturized low-cost ion mobility spectrometer for fast detection of chemical warfare agents. *Anal. Chem.* **2008**, *80*, 6671–6676.

[67] Environics Oy: ChemProX, https://environics.fi/products/chemprox/ (accessed 2 December 2021).

[68] Maruko, H.; Sekiguchi, H.; Seto, Y.; Sato, A. Comparison of chemical agent detection performance of portable aspiration-type ion mobility spectrometer devices. *Bunseki Kagaku* **2006**, *55*, 191–197.

[69] Kishi, S.; Sekioka, R.; Sodeyama, M.; Shiga, M.; Seto, Y. Comparison of chemical agent detection performance of aspiration-type ion mobility spectrometer devices. *Bunseki Kagaku* **2010**, *59*, 65–76.

[70] Smiths Detection: Chemical agent detection, https://www.smithsdetection.com/product-finder/chemical-detection-or-identification (accessed 2 December 2021).

[71] Sato, T.; Kishi, S.; Nagashima, H.; Tachikawa, M.; Kanamori-Kataoka, M.; Nakagawa, T.; Kitagawa, N.; Tokita, K.; Yamamoto, S.; Seto, Y. Ion mobility spectrometric analysis of vaporous chemical warfare agents by the portable instrument with corona discharge ionization ammonia dopant ambient temperature operation. *Anal. Chim. Acta* **2015**, *865*, 38–52.

[72] Yamaguchi, S.; Asada, R.; Kishi, S.; Sekioka, R.; Kitagawa, N.; Tokita, K.; Yamamoto, S.; Seto, Y. Detection performance of a portable ion mobility spectrometer with ^{63}Ni radioactive ionization for chemical warfare agents. *Forensic Toxicol.* **2010**, *28*, 84–95.

[73] Utriainen, M; Kärpänoja, E.; Paakkanen, H. Combining miniaturized ion mobility spectrometer and metal oxide gas sensor for the fast detection of toxic chemical vapors. *Sens. Actuat. B* **2003**, *93*, 17–24.

[74] AIRSENSE Analytics: GDA Fumigation, https://airsense.com/en/products/gda-fumigation (accessed 2 December 2021).

[75] Fallman, A.; Rettfeldt, L. Detection of chemical warfare agents in water by high temperature-solid phase microextraction-ion mobility spectrometry (HT-SPME-IMS). *Int. J. Ion. Mobil. Spectrom.* **2001**, *4*, 85–87.

[76] Mukhopadhyay, R. Product Review: Portable FTIR spectrometers get moving. *Anal. Chem.* **2004**, *76*, 369A–372A.

[77] Gasmet Technologies Oy: Portable Gas Analyzers https://www.gasmet.com/products/category/portable-gas-analyzers/ (accessed 2 December 2021).

[78] JASCO: VIR-200, https://www.jasco-global.com/product-spectroscopy/ftir/ (accessed 2 December 2021).

[79] Smiths Detection: HazMatID Elite, https://www.smithsdetection.com/products/hazmatid-elite/ (accessed 2 December 2021).

[80] Thermo Scientific: FirstDefender RM, https://www.thermofisher.com/order/catalog/product/FIRSTDEFENDERRM (accessed 2 December 2021).

[81] Rigaku Corporation: Progeny ResQ, https://www.rigaku.com/products/raman/resq-chemical-

---

analyzer (accessed 2 December 2021).

[82] O.I. Analytical: GC Air Monitoring Instrumentation, https://www.oico.com/products/gc-continuous-monitoring-systems (accessed 2 December 2021).

[83] Buryakov, I. A. Express analysis of explosives, chemical warfare agents and drugs with multicapillary column gas chromatography and ion mobility increment spectrometry. *J. Chromatogr. B* **2004**, *800*, 75–82.

[84] GAS: GC–IMS, https://www.gas-dortmund.de/Products/Instruments/GC-IMS/1_367.html (accessed 2 December 2021).

[85] Williams, D.; Pappas, G. Rapid identification of nerve agents sarin (GB) and soman (GD) with the use of a field-portable GC/SAW vapor detector and liquid desorption front-end device. *Field Anal. Chem. Technol.* **1999**, *69*, 45–53.

[86] Wise, M. B.; Guerin, M. R. Peer Reviewed: Direct sampling ms for environmental screening. *Anal. Chem.* **1997**, *69*, 26A–32A.

[87] Virkki, V. T.; Ketola, R. A.; Ojala, M.; Kotiaho, T.; Komppa, V.; Grove, A.; Facchetti, S. On-Site Environmental Analysis by Membrane Inlet Mass Spectrometry *Anal. Chem.* **1995**, *67*, 1421–1425.

[88] Kidera, M.; Seto, Y.; Takahashi, K.; Enomoto, S.; Kishi, S.; Makita, M.; Nagamatsu, T.; Tanaka, T.; Toda, M. New method for comprehensive detection of chemical warfare agents using an electron-cyclotron-resonance ion-source mass spectrometer. *Spectrochim. Acta A* **2011**, *78*, 437–444.

[89] Urabe, T.; Takahashi, K.; Kitagawa, M.; Sato, T.; Kondo, T.; Enomoto, S.; Kidera, M.; Seto, Y. Development of portable mass spectrometer with electron cyclotron resonance ion source for detection of chemical warfare agents in air. *Rapid Commum. Mass Spectrom.* **2011**, *25*, 1437–1444.

[90] Nilles, J. M.; Connell, T. R.; Durst, H. D. Quantitation of Chemical Warfare Agents Using the Direct Analysis in Real Time (DART) Technique. *Anal. Chem.* **2009**, *81*, 6744–6749.

[91] Iwai, T.; Kakegawa, K.; Okumura, K.; Kanamori-Kataoka, M.; Miyahara, H.; Seto, Y.; Okino, A. Fundamental properties of a touchable high-power pulsed microplasma jet and its application as a desorption/ionization source for ambient mass spectrometry. *J. Mass Spectrom.* **2014**, *49*, 522–528.

[92] Henry, C. Taking the show on the road: Portable GC and AC/MS. *Anal. Chem.* **1997**, *69*, 195A–201A.

[93] Eckenrode, B. A. Environmental and Forensic Applications of Field-Portable GC–MS: An Overview. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 683–693.

[94] Makas, A. L.; Troshkov, M. L. Field gas chromatography–mass spectrometry for fast analysis. *J. Chromatogr. B* **2004**, *800*, 55–61.

[95] Smith, P. A.; Sng, M. T.; Eckenrode, B. A.; Leow, S. Y.; Koch, D.; Erickson, R. P.; Jackson Lepage, C. R.; Hook, G. L. Towards smaller and faster gas chromatography–mass spectrometry systems for field chemical detection. *J. Chromatogr. A* **2005**, *1067*, 285–294.

[96] Smith, P. A.; Koch, D.; Hook, G. L.; Erickson, R. P.; Jackson Lepage, C. R.; Wyatt, H. D. M.; Betsinger, G.; Eckenrode, B. A. Detection of gas-phase chemical warfare agents using field-portable

---

gas chromatography–mass spectrometry systems: instrument and sampling strategy considerations. *Trend. Anal. Chem.* **2004**, *23*, 296–306.

[97] Sekiguchi, H.; Matsushita, K.; Yamashiro, S.; Sano, Y.; Seto, Y.; Okuda, T.; Sato, A. On-site determination of nerve gases and mustard gas using field-portable gas chromatograph–mass spectrometer. *Forensic. Toxicol.* **2006**, *24*, 17–22.

[98] INFICON: Hapsite ER, https://www.inficon.com/de/produkte/hapsite-er-identification-system (accessed 2 December 2021).

[99] Smith, P. T. Person-portable gas chromatography: Rapid temperature program operation through resistive heating of columns with inherently low thermal mass properties. *J. Chromatogr. A* **2012**, *1261*, 37–45.

[100] Kwak, J.; Fan, M.; Geier, B. A. Grigsby, C. C.; Ott, D. K. Comparison of sampling probe and thermal disorder in HAPSITE ER for analysis of TO-15 compounds *J. Anal. Bioanal. Tech.* **2014**, S2–S5.

[101] PerkinElmer: Torion T-9 GC/MS, https://www.perkinelmer.com/product/torion-t-9-portable-gc-ms-instrument-ntsst090500 (accessed 2 December 2021).

[102] Contreras, J. A.; Murray, J. A.; Tolley, S. E.; Oliphant, J. L.; Tolley, H. D.; Lammert, S. A.; Lee, E. D.; Later, D. W.; Lee, M. L. Hand-portable gas chromatograph-toroidal ion trap mass spectrometer (GC-TMS) for detection of hazardous compounds. *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 1425–1434.

[103] Smith, P. A.; Jackson Lepage, G. R.; Savage, P. B.; Bowerbank, C. R.; Lee, E. D. Lukacs, M. J. Use of a hand-portable gas chromatograph–toroidal ion trap mass spectrometer for self-chemical ionization identification of degradation products related to *O*-ethyl *S*-(2-diisopropylaminoethyl) methyl phosphonothiolate (VX). *Anal. Chim. Acta* **2011**, *690*, 215–220.

[104] Zhang, S.-W.; Swager, T. M. Fluorescent Detection of Chemical Warfare Agents: Functional Group Specific Ratiometric Chemosensors. *J. Am. Chem. Soc.* **2003**, *125*, 3420–3421.

[105] Khan, M. A. K.; Kerman, K.; Petryk, M.; Kraatz, H. B. Noncovalent Modification of Carbon Nanotubes with Ferrocene−Amino Acid Conjugates for Electrochemical Sensing of Chemical Warfare Agent Mimics. *Anal. Chem.* **2007**, *80*, 2574–2582.

[106] Riken Keiki Co. Ltd.: Portable single gas, https://product.rikenkeiki.co.jp/lp/en/med/ (accessed 2 December 2021).

[107] Matsuura, H.; Yamada, H.; Asada, R.; Yamaguchi, S.; Sato, K.; Nakano, N.; Nishiyama, K.; Taniguchi, I.; Seto, Y. Amperometric Sensor Consisting of Gold-Nanoparticle-Dispersed Carbon Fiber Electrodes for Blister Agent Detection. *Sens. Mater.* **2010**, *22*, 167–174.

[108] Russell, R. J.; Pishko, M. V.; Simonian, A. L.; Wild, J. R. Poly(ethylene glycol) Hydrogel-Encapsulated Fluorophore−Enzyme Conjugates for Direct Detection of Organophosphorus Neurotoxins. *Anal. Chem.* **1999**, *71*, 4909–4912.

[109] Wang, J. Microchip devices for detecting terrorist weapons. *Anal. Chim. Acta.* **2004**, *507*, 3–10.

[110] Anzai, J. *Biosensors for the detection of OP nerve agents. in: Handbook of the Toxicology of*

Chemical Warfare Agents (Gupta, R. C. eds.), 2nd ed., Chapter 62, pp. 925–934, Elsevier, Amsterdam, 2015.

[111] Zayats, M.; Kharitonov, A. B.; Pogorelova, S. P.; Lioubashevski, O.; Katz, E.; Willner, I. Probing Photoelectrochemical Processes in Au−CdS Nanoparticle Arrays by Surface Plasmon Resonance: Application for the Detection of Acetylcholine Esterase Inhibitors. *J. Am. Chem. Soc.* **2003**, *125*, 16006–16014.

[112] Palleschi, G.; Bernabei, M.; Cremisini, G.; Mascini, M. Determination of organophosphorus insecticides with a choline electrochemical biosensor. *Sens. Actuat. B* **1992**, *7*, 513–517.

[113] Guerrieri, G.; Monaci, L.;Quinto, M.; Palmesano, F. A disposable amperometric biosensor for rapid screening of anticholinesterase activity in soil extracts. *Analyst* **2005**, *127*, 5–7.

[114] Walker, J. P.; Asher, S. A. Acetylcholinesterase-Based Organophosphate Nerve Agent Sensing Photonic Crystal. *Anal. Chem.* **2005**, *77*, 1596–1600.

[115] Karnati, C.; Du, H.; Ji, H.-F.; Xu, X.; Lvov, Y.; Mulchandani, A.; Mulchandani, P.; Chen, W. Organophosphorus hydrolase multilayer modified microcantilevers for organophosphorus detection. *Biosens, Bioelectron.* **2007**, *22*, 2636–2642.

[116] Shan, D.; Mousty, C.; Cosnier, S. Subnanomolar Cyanide Detection at Polyphenol Oxidase/Clay Biosensors. *Anal. Chem.* **2004**, *76*, 178–183.

[117] Vanininen, P., *Recommended Operating Procedures for Sampling and Analysis in the Verification of Chemical Disarmament*, University of Helsinki, Helsinki, 2017.

[118] Hooijschuur, E. W. J.; Kientz, C. E.; Brinkman, U, A, T, Analytical separation techniques for the determination of chemical warfare agents. *J. Chromatogr. A* **2002**, *982*, 177–200.

[119] Black, R. M.; Muir, B. Derivatisation reactions in the chromatographic analysis of chemical warfare agents and their degradation products. *J. Chromatogr. A* **2003**, *1000*, 253–281.

[120] Tsunoda, N. Seto, Y. *Rept. Natl. Res. Inst. Police Sci.* **1997**, *50*, 59–80.

[121] Ando, H.; Miyata, Y., *Drugs and Poisons in Human—A Handbook of Practical Analysis (Suzuki, O.; Watanabe, K. eds.),* pp. 609–618, Springer, Berlin, 2005.

[122] Katagi, M.; Tsuchihashi, H., *Drugs and Poisons in Human—A Handbook of Practical Analysis (Suzuki, O.; Watanabe, K. eds.)*, pp. 619–627, Springer, Berlin, 2005.

[123] Hanaoka, N., *Drugs and Poisons in Human—A Handbook of Practical Analysis (Suzuki, O.; Watanabe, K. eds.),* pp. 69–90, Springer, Berlin, 2005.

[124] Kataoka, M.; Tsunoda, N.; Ohta, H.; Tsuge, K.; Takesako, H.; Seto, Y. Effect of cation-exchange pretreatment of aqueous soil extracts on the gas chromatographic–mass spectrometric determination of nerve agent hydrolysis products after *tert*.-butyldimethylsilylation. *J. Chromatogr. A* **1998**, *824*, 211–221.

[125] Kaise, T.; Kinoshita, K. Analysis of organic arsenic compounds detected in groundwater and their toxicity. *Bunseki*, **2005**, 284–289.

[126] Read, R. W.; Black, R. M. Analysis of β-Lyase Metabolites of Sulfur Mustard in Urine by

---

Electrospray Liquid Chromatography-Tandem Mass Spectrometry. *J. Anal. Toxicol.* **2004**, *28*, 346–351.

[127] Tsuge, K.; Seto, Y. Analysis of organophosphorus compound adducts of serine proteases by liquid chromatography–tandem mass spectrometry. *J. Chromatogr. B* **2002**, *776*, 79–88.

[128] Tsuge, K.; Seto, Y. Detection of human butyrylcholinesterase-nerve gas adducts by liquid chromatography–mass spectrometric analysis after in gel chymotryptic digestion. *J. Chromatogr. B* **2006**, *838*, 21–30.

[129] Polhuijs, M.; Langenberg, J. P.; Benschop, H. P. New method for retrospective detection of exposure to organophosphorus anticholinesterases: application to alleged sarin victims of Japanese terrorists, *Toxicol. Appl. Pharmacol.* **1997**, *146*, 156–161.

[130] Seto, Y.; Tachikawa, M.; Kanamori-Kataoka, M.; Sasamoto, K.; Ochiai, N. Gas chromatography-mass spectrometry with spiral large-volume injection for determination of fluoridated phosphonates produced by fluoride-mediated regeneration of nerve agent adduct in human serum. *J. Chromatogr. A* **2019**, *1583*, 108–116.

Chapter 2

A Screening Method for Cyanide in Blood by Dimethoxytriazinyl Derivatization-GC/MS

Chapter 2 A Screening Method for Cyanide in Blood by Dimethoxytriazinyl Derivatization-GC/MS

2-1. Introduction

Cyanide analysis is of forensic importance because cyanide is a compound with severe, acute toxicity in blood, and thus it is sometimes involved in intentional poisoning. Cyanide has been used in recent serial homicides in Japan and India.[1,2] Accordingly, analysis of cyanide in autopsy samples is essential to prevent crime-related death from being overlooked.[1] However, the cyanide ion has high polarity and low molecular mass, and thus difficult to detect by general screening method for unknowns using liquid chromatography-mass spectrometry.

For the sensitive analysis of cyanide, many methods have been developed using colorimetric[3,4,5] or fluorimetric[6,7] detection, or specific electrodes.[8] These methods require microdiffusion pretreatment, which isolates volatile cyanide from non-volatile interfering species using a Conway cell.[9,10] Moreover, in forensic toxicological analysis, mass spectrometry is preferred due to its high sensitivity and accuracy. Accordingly, headspace gas chromatography/mass spectrometry (GC/MS)[11] has become popular for cyanide analysis due to its easy operation and high accuracy. Alternatively, a liquid chromatography-tandem mass spectrometry method was developed in which cyanide was derivatized with taurine and 2,3-naphthalenedialdehyde.[12,13] In addition, pentafluorobenzyl (PFB) derivatization-GC/MS is widely used due to the simple procedure,[14] which is useful for field forensic toxicologists. However, pentafluorobenzyl bromide, the reagent for the PFB derivatization, is a potent lachrymator, and the derivatization requires heating for 30 min and a phase transfer catalyst, which can damage the capillary column.[14] Therefore, a simpler, faster method is required by field forensic toxicologists.

We focus on 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM), which is commercially available as a condensing agent in synthetic organic chemistry. DMTMM activates the carboxylic acid in dehydration condensation reactions with amines at room temperature in water and alcohol.[15] The suitability of water as a solvent is particularly desirable in the derivatization reaction of cyanide, which is almost insoluble in organic solvents. Moreover, no phase transfer catalyst is required,[14] which can damage gas chromatography columns. Furthermore, because DMTMM is an inexpensive stable solid with low irritation and toxicity we expected that it could be easily used for screening.

Kakehashi et al. reported a method for derivatizing cyanide in beverages and blood with DMTMM; however, the method focuses on screening for cyanide in beverages, and thus the validated concentration points for quantitative analysis were 30 and 300 µg/m.[16] The detection limit of cyanide in blood by their method was 0.5 µg/mL, which cannot fully cover blood samples from poisoning cases (0.1–0.2 µg/mL.[17]

We have independently discovered the reaction between DMTMM and cyanide forming 2-cyano-4,6-dimethoxy-1,3,5-triazine (DMTCN, Figure 2-1) and optimized this method for screening biological samples to cover blood samples from poisoning cases. Here, we report a simple screening analysis of cyanide in blood using DMTMM. The derivatization conditions were optimized for aqueous solutions and cyanide-spiked blood samples. The optimized method was verified by intra-day and inter-day repetition analyses of spiked blood samples (cyanide 0.1–5 μg/mL).

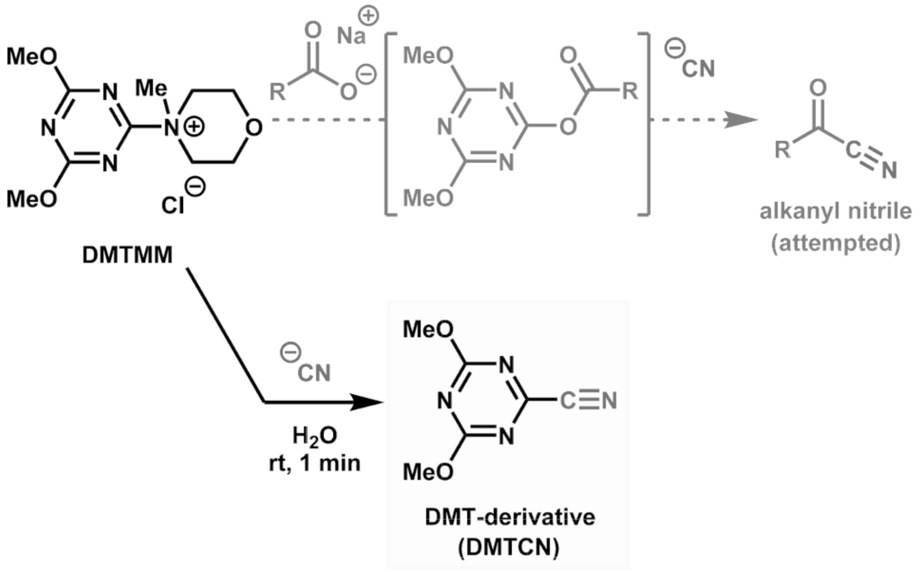


Figure 2-1. The present derivatization reaction of cyanide ion with DMTMM (black), and the attempted formation of alkanyl nitrile via dehydrocondensation (gray).

2-2. Experimental

2-2-1. Materials

Cyanide-spiked aqueous solution and blood containing cyanide were prepared using potassium cyanide (Kanto Chemical Co., Inc., Tokyo, Japan). NaOH (Wako Pure Chemicals Co., Ltd., Osaka, Japan), ultrapure water (Organo Co., Ltd., Tokyo, Japan), and reference blood not containing HCN (blank blood) for other than the determination of method selectivity (BIOPREDIC International, Rennes, France) were purchased. Derivatization reagent, DMTMM (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), was adjusted to a 20% (w/v) aqueous solution. The internal standard compound (IS), 1,3,5-tribromobenzene (TBB; Tokyo Chemical Industry), was adjusted to 10 μmol/L solution in ethyl acetate (EtOAc). Sodium tetraborate decahydrate (borax; Wako Pure Chemicals), special grade EtOAc (Kanto Chemical), and trichloroacetic acid (TCA; Kanto Chemical) were used for reactions. The other reagents were purchased from Wako Pure Chemicals, Kanto Chemical or Tokyo Chemical Industry.

Blank blood specimens for the determination of method selectivity were collected from healthy volunteers with informed consent, by nurses using vacuum vessels containing EDTA-2K with informed consent and were stored in a freezer until analysis. This study was conducted with ethical approval by the Research Ethics Committee of the National Research Institute of Police Science, Japan.

2-2-2. Instrumentation

A gas chromatography-mass spectrometer (GC-MS) SHIMADZU QP2020NX (Shimadzu Corporation, Kyoto, Japan) was used to analyze the samples. The GC column used was Agilent Technologies DB-5ms (30 m×0.25 mm, 0.25 µm, Agilent Technologies, Santa Clara, CA, USA). The inlet liner used was SHIMADZU 95 mm, deactivated, with wool for Split and Splitless (volume: 862 µL, parts number: 221-75193, Shimadzu Corporation). The GC-MS conditions were as follows: carrier gas: helium (1.0 mL/min), oven program: 60 °C (3 min) –25 °C/min–285 °C (3 min), injection volume: 1 µL, split ratio: 2:1, ionization: Electron Ionization (EI), scan range: *m/z* 50–500, extracted ion: *m/z* 136 (DMTCN, target), 312 (TBB, IS).

2-2-3. Sample preparation (aqueous solution)

Borax (30 mg, for removal of hydration water of cyanide and salting out of DMTCN) and the sample (200 µL) were added to a microtube before adding DMTMM solution (100 µL, 20 w/v %). The mixture was vortexed for 1 min at 23 °C, and then TBB solution (400 µL, 10 µmol/L in EtOAc, selected as the IS because of its low reactivity and appropriate retention time) was added and the mixture was vortexed again. After centrifugation (3000×*g*, 10 s at 23 °C), the supernatant was used as the test sample for the analysis.

2-2-4. Sample preparation (blood)

Cold TCA aq. (200 µL, 10 w/v %, 0 °C, for protein precipitation) was added to the blood sample (200 µL) in a microtube. The mixture was vortexed for 20 s and centrifuged (13,000×*g*, 5 min at 4 °C). The supernatant (200 µL) was transferred to another microtube and saturated borax aq. (800 µL) was added, followed by adding DMTMM solution (200 µL, 20 w/v %). The mixture was stirred for 1 min at 23 °C, and then TBB solution (400 µL, 10 µmol/L in EtOAc) was added and the mixture was vortexed. After centrifugation (3000×*g*, 10 s at 23 °C), the supernatant was transferred to a gas chromatography vial for the analysis.

An equivalent of 100 µL blood was in the final extract, or 2× diluted compared to the aqueous solution.

2-2-5. Validation procedures

Selectivity, linearity, sensitivity, accuracy (systematic error) and precision (random error) were evaluated according to the United States Food and Drug Administration guidelines.[18]

2-2-5-1. Determination of method selectivity and interference

The method selectivity was evaluated by using five different blood samples as matrices. In addition, each sample without either cyanide or TBB was analyzed to assess potential interference. The criteria that target peaks do not overlap with peaks derived from endogenous matrices was that there was neither a peak with area of more than 5% of the TBB peak area nor more than 20% of the analyte peak area at the lower limit of quantitation (LLOQ) at the retention time of the target. To evaluate the method selectivity in the presence of other anions, 14 anion species (CN^-, SCN^-, NO_2^-, N_3^-, HS^-, CH_3COO^-, F^-, Cl^-, Br^-, I^-, HCO_3^-, NO_3^-, $S_2O_3^{2-}$, SO_4^{2-}) were tested using spiked blood samples of each anion (CN^- 1 µg/mL, other anions 5 µg/mL). Each blood sample was pretreated, and the presence of a peak at the retention time for DMTCN (8.03 min) on the extracted ion chromatogram (EIC; *m/z* 136) was examined. To evaluate the interferences, competition experiments were performed in which blood samples containing CN^- (1 µg/mL) and each anion (5 µg/mL, the same anions as used above) were used.

2-2-5-2. Evaluation of linearity, sensitivity, intra- and inter-day accuracy and precision

To evaluate linearity, sensitivity, accuracy and precision, calibration curves were constructed using spiked 0.1 mol/L NaOH aqueous solution and whole blood samples at six or seven concentrations. The LLOQ was determined as the lowest concentration at which both accuracy and precision were less than or equal to 20%. The intra- and inter-day accuracy and precision for the standard samples should be within 15%, except at the LLOQ, at which a value of 20% is deemed acceptable.

2-3. Results

2-3-1. Optimization of derivatization condition

We first attempted alkanyl nitrile formation with DMTMM (Figure 2-1). However, this condensation reaction hardly proceeded. Instead, DMTCN (Figure 2-1), in which the morpholinyl group of DMTMM was replaced with a nitrile group, was produced. Because DMTCN was formed quantitatively in an aqueous one-phase system at room temperature after 1 min, we focused on DMTCN as the target for derivatization GC/MS analysis of cyanide. Owing to the simplicity of the methods, general purpose GC-MS settings, including those for inlets and columns, were used.

The amount of DMTMM was set to 20 mg for 0.1 mol/L NaOH and 40 mg for blood, based on a balance between reagent usage and reaction efficiency (ratio of DMTMM amount to peak area of DMTCN of: (a) 0.1 mol/L NaOH: 10 mg : 20 mg : 40 mg = 68% : 100% : 116%, (b) blood: 20 mg : 40 mg : 80 mg = 65% : 100% : 119%). In each sample (cyanide 1 µg/mL), the derivative was efficiently generated at room temperature after 1 min.

To extract DMTCN from the aqueous phase, we added ethyl acetate after the derivatization reaction because of the verification results for the extraction efficiency (before reaction : after reaction = 89% : 100%, based on peak area of DMTCN obtained from 0.1 mol/L NaOH, cyanide 1 µg/mL).

We also found that adding borax improved the reaction efficiency (borax 0 mg : borax 30 mg = 39% : 100%, based on peak area of DMTCN obtained from 0.1 mol/L NaOH, cyanide 1 µg/mL), which is consistent with the observations of Kakehashi et al..[16] This improvement was not observed when sodium bicarbonate was added (data not shown), so this effect was probably caused by the removal of hydration water of cyanide and salting out of DMTCN by borate ion, which is highly kosmotropic.[19]

A reaction time of 1 min was considered to be sufficient (1 min : 5 min : 10 min : 20 min = 100% : 104% : 99% : 84%, based on peak area of DMTCN obtained from 0.1 mol/L NaOH, cyanide 1 µg/mL).

We determined that the derivatization reaction yield of cyanide in 0.1 mol/L NaOH was 44 ± 5% by comparing DMTCN synthesized in-house,[20] which was comparable to the yield of conventional PFB-derivatization of cyanide (40%, data not shown).

2-3-2. Modification for blood analysis

Protein precipitation for whole blood with TCA was introduced to reduce negative influences by blood constituents. The TCA treatment efficiently improved derivatization efficiency, whereas dilution alone used in a previous study[16] provided poor derivatization efficiency (Table 2-1, entry 1). Adding 200 µL of the supernatant obtained from 10% TCA-precipitation to 800 µL of saturated borax aqueous solution (Table 2-1, entry 3) gave good results (Table 2-1, entries 2–6).

Table 2-1. Optimization of blood pretreatment condition

entry	pretreatment of blood			DMTCN/TBB (peal area)[a]
	sample (µL)	[TCA] (w/v %)	sat. borax aq. (µL)	
1	100	-	900	6.6%
2	200	20	800	81.6%
3	200	10	800	100.0%
4	200	10	800[b]	93.5%
5	200	10	400	76.1%
6	200	10	-[c]	19.2%

- : not added., [a] Set entry 3 as 100.0%., [b] Borax 30 mg was added.,
[c] Sat. $NaHCO_3$ aq. 800 µL was added.

2-3-3. Chromatograph and mass spectrum

The chromatograms obtained from 1 µg/mL cyanide in 0.1 mol/L NaOH, which is equivalent to a sample after microdiffusion, and from whole blood containing cyanide (1 µg/mL) are shown in Figure 2-2A, B. The peaks at retention times of 8.03 and 8.70 min correspond to DMTCN and TBB, respectively (Figures 2-2A, B). In addition, EIC (*m/z* 136, base peak) and mass spectra of the standard DMTCN are also shown in Figures 2-2C, D, respectively.

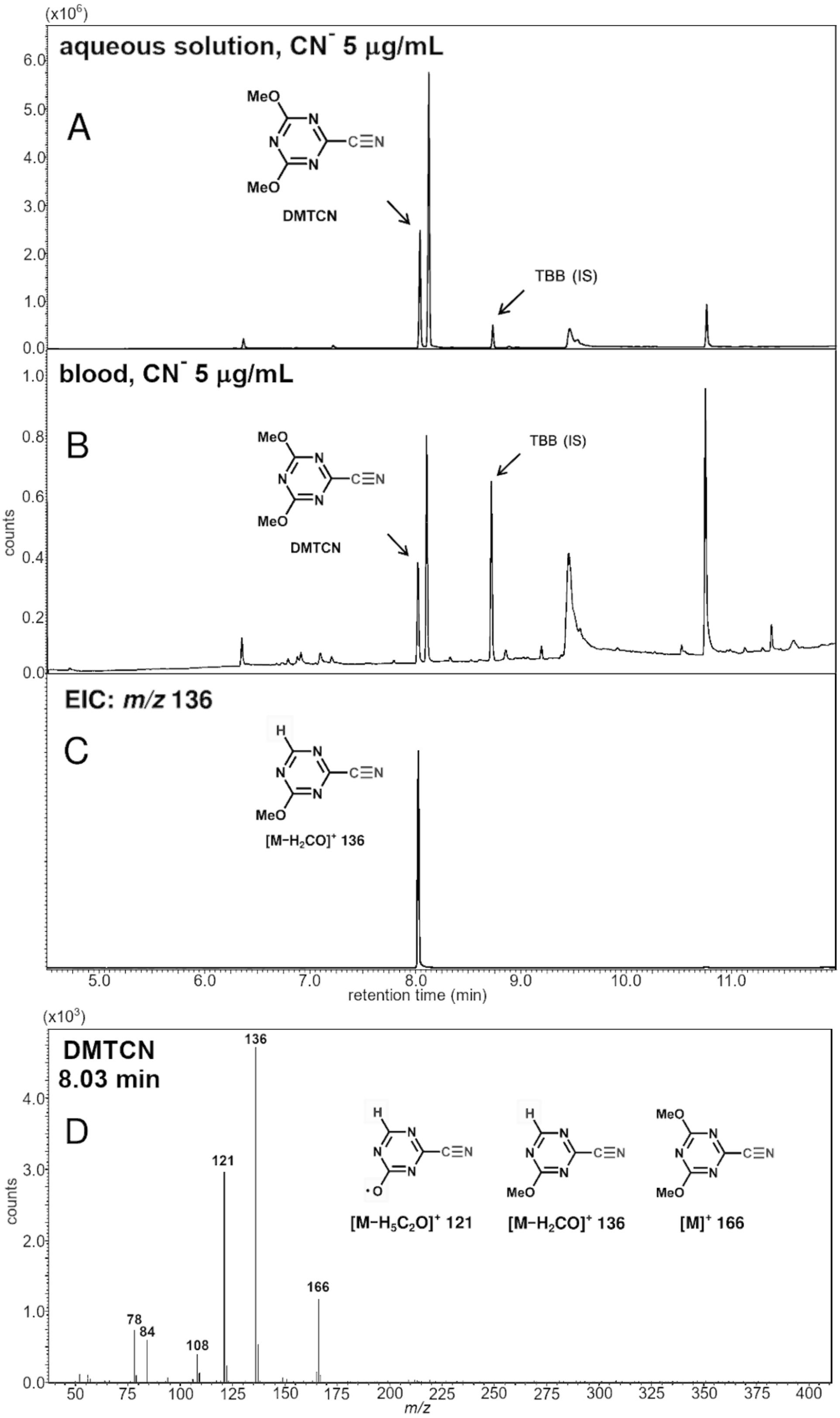


Figure 2-2. (A), (B) Total ion current chromatogram obtained from (A) 0.1 mol/L NaOH, (B) blood, (C) EIC (*m/z* 136) of standard DMTCN in EtOAc, (D) EI mass spectrum of DMTCN.

2-3-4. Validation

2-3-4-1. Linearity and sensitivity

When this method was performed on blood specimens obtained from five healthy non-smokers, there were neither a peak with area of more than 5% of the TBB peak area nor more than 20% of the analyte peak area at the LLOQ at or near the retention time of the target, and there were no minor peaks in the each extracted ion chromatogram (data not shown). Therefore, the analyzed peaks did not overlap with peaks derived from endogenous matrices. Linearity and sensitivity were determined to be as follows: aqueous solution: regression equation: $Y = 1.6393X-0.0231$, $R^2 = 0.990$, blood: regression equation: $Y = 0.6980X-0.0331$, $R^2 = 0.987$.

2-3-4-2. Intra- and inter-day accuracy and precision

The results of repeated intra- and inter-day analysis (0.05, 0.1, 0.25, 1 and 5 µg/mL, *n* = 5) using the calibration curves prepared for the aqueous solution and whole blood are shown in Table 2-2.

The concentration ranges, which are compatible with the US Food and Drug Administration guidelines (accuracy and precision within 20% for the lower limit of quantification and within 15% for other concentration[18]), were 0.05–5 µg/mL for the aqueous solution and 0.1–5 µg/mL for whole blood. Thus, the LLOQ of aqueous solution and blood were 0.05 and 0.1 µg/mL, respectively. The limit of detection (signal-to-noise ratio ≥3) of the aqueous solution and blood was 0.01 and 0.05 µg/mL, respectively (Figure 2-3A, B).

Table 2-2. Validation results

sample (*n* = 5)			[cyanide] (µg/mL)				
			0.05	0.1	0.25	1	5
aqueous solution	intra-day	accuracy (%)	8.3	-6.1	-9.9	-7.7	6.7
		precision (%)	14.5	11.3	3.7	7.4	2.2
	inter-day	accuracy (%)	5.9	-2.5	-13.9	-14.3	-1.9
		precision (%)	6.9	5.3	9.7	10.3	10.9
blood	intra-day	accuracy (%)	-	7.7	-14.2	-3.4	4.7
		precision (%)	-	1.7	7.2	1.9	5.8
	inter-day	accuracy (%)	-	3.5	-12.9	-13.6	14.8
		precision (%)	-	18.6	10.8	13.7	3.6

- : not tested.

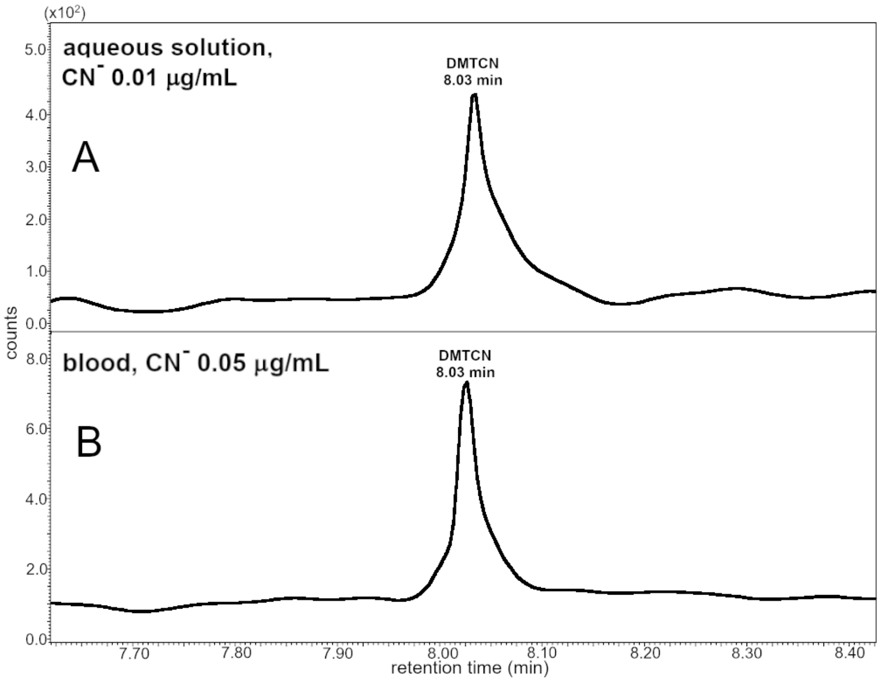


Figure 2-3. EIC (*m/z* 136) obtained from (A) 0.1 mol/L NaOH (cyanide 0.01 μg/mL), (B) blood (cyanide 0.05 μg/mL).

2-3-4-3. Selectivity and interference

In the selectivity test, only CN^- yielded DMTCN (Figure 2-4, seven of these results and a blank blood result are shown). No DMTCN peaks were detected even in the case of SCN^-, NO_2^- and NO_3^-, which are known to yield cyanide and show false positives by acid treatment of blood samples,[21] probably because of the difference in pK_a between acids used: H_2SO_4 (pK_a -3, used in the same amount of 10% solution as blood in a usual acid treatment), TCA (pK_a 0.66, used in the same amount of 10% solution as blood in the present method).

In the interference test, there was no significant interference (no ≥8% increase or decrease in the peak area of DMTCN).

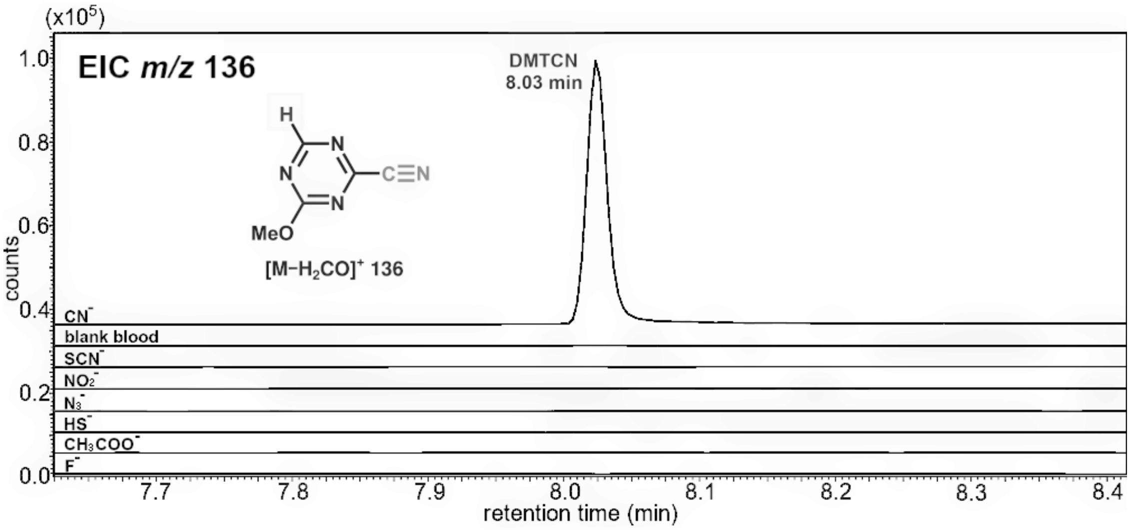


Figure 2-4. EIC (*m/z* 136) obtained from cyanide (1 µg/mL) in blood, blank blood, other anions (5 µg/mL) in blood.

2-4. Discussion

From what has been shown above, we can conclude that samples containing cyanide at concentrations ranging from toxic (0.1–0.2 µg/mL) to lethal (3–5 µg/mL) can be used not only for quantitative analysis after microdiffusion, but also for screening analysis of cyanide by simple pretreatment of blood. The selectivity and interference results demonstrated that this GC-MS method had selectivity towards cyanide ion and robustness against other anions. The present method has comparable analytical performance to PFB-derivatization without the disadvantages of lachrymatory properties, long reaction time, and heatin.[14] In addition, the present method achieved the analysis of blood cyanide in the toxic concentration range, which the previous study using DMTMM did not.[16] Moreover, the derivatization reaction required 20 min stirring in the previous study, whereas 1 min stirring was sufficient in our optimized method.

2-5. References

---

[1] https://www.sankei.com/smp/west/news/150128/wst1501280029-s.html '[Hydrocyanic acid serial killer] "Kyoto" found the case a serial murder. Chisako defendant received insurance money and condominium for inner-law men' *Sankei Shinbun* 28 Jan. 2015.

[2] https://www.nytimes.com/2019/10/19/world/asia/india-koodathayai-murder.html 'Police Say Cyanide Soup and 6 Deaths Point to a Serial Killer' *The New York Times* 19 Oct. 2019.

[3] Feldsteinand, M., Klendshoj, N. C.; The determination of cyanide in biologicfluids by microdiffusion analysis; *J. Lab. Clin. Med.*, **1954**, *44*, 166–170.

[4] Lundquist, P., Rosling, H., Sörbo. B.; Determination of cyanide in whole blood, erythrocytes, and plasma; *Clin. Chem.*, **1985**, *31*, 591–595.

[5] Laforge, M., Buneaux, F., Houeto, P., Bourgeois, F., Bourdon, R., Levillain, P.; A rapid spectrophotometric blood cyanide determination applicable to emergency toxicology; *J. Anal. Toxicol.*, **1994**, *18*, 173–175, doi: 10.1093/jat/18.3.173

[6] Morgan, R. L., Way, J. L.; Fluorimetric determination of cyanide in biologicalfluids with pyridoxal; *J. Anal. Toxicol.*, **1980**, *4*, 78–81, doi: 10.1093/jat/4.2.78

[7] Ganjeloo, A., Isom, G. E., Morgan, R. L., Way, J. L.; Fluorometric determination of cyanide in biologicalfluids with p-benzoquinone; *Toxicol. Appl. Pharmacol.*, **1980**, *55*, 103–107.

[8] Kistner, J. R., Longnecker, D. E., Miller, E. D. Jr., Lescanic, A. D.; Limitations of the cyanide electrode for use in plasma and whole blood; *Anesth. Analg.*, **1979**, *58*, 457–460.

[9] Edward, J. C.; Microdiffusion Analysis and Volumetric Error; *Nature*, **1948**, *161*, 583. 10.1038/161583a0

[10] Okada, Y., Miyaguchi, H.; Development of a handy microdiffusion device using two plastic test tubes for accurately quantifying cyanide in blood; *Forensic Toxicol.* **2020**, *38*, 542–546, doi: 10.1007/s11419-020-00536-9

[11] Odoul, M., Fouillet, B., Nouri, B., Chambon, R., Chambon, P.; Specific determination of cyanide in blood by headspace gas chromatography; *J. Anal. Toxicol.*, **1994**, *18*, 205–207, doi: 10.1093/jat/18.4.205

[12] Lai, F., Sheehan. T.; Matrix effect in the derivatization of amino acids with naphthalene dicarboxyaldehyde, 9-fluorenylmethyl chloroformate and phenylisothiocyanate; *Biotechniques*, **1993**, *14*, 642–649.

[13] Lacroix, C., Saussereau, E., Boulanger, F., Goullé, J. P.; Online Liquid Chromatography-Tandem Mass Spectrometry Cyanide Determination in Blood; *J. Anal. Toxicol.*, **2011**, *35*, 143–147, doi: 10.1093/anatox/35.3.143

[14] Kage, S., Nagata, T., Kudo. K.; Determination of cyanide and thiocyanate in blood by gas chromatography and gas chromatography–mass spectrometry; *J. Chromatogr. B* **1996**, *675*, 27–32, doi: 10.1016/0378-4347(95)00344-4

---

[15] Kunishima, M., Kawachi, C., Monta, J., Terao, K., Iwasaki, F., Tani, S.; 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride: an efficient condensing agent leading to the formation of amides and esters; *Tetrahedron* **1999**, *55*, 13159─13170, doi: 10.1016/S0040-4020(99)00809-1

[16] Kakehashi, H., Shima, N., Kamata, H., Nishioka, H., Ishikawa, A., Asai, R., Nitta, A., Wada, M., Nakano, S., Matsuta, S., Sasaki, K., Kamata, T., Miki, A., Katagi, M.; Development of new method for cyanide determination using dimethoxytriazinyl (DMT) derivertization; *Jpn. J. Forensic Sci. Tech.* **2020**, *25*, 141–150, doi: 10.3408/jafst.774 [in Japanese]

[17] Stead, A. H., Moffat, A. C.; A Collection onf Therapeutic, Toxic and Fatal Blood Drug Concentrations in Man; *Human Toxicol.* **1983**, *3*, 437–464.

[18] U.S. Department of Health and Human Services, Food and Drug Administration, Guidance for Industry: Bioanalytical method validation, 2013.

[19] Aggarwal, N.; Interactions between Saccharides and Phosphate-based Salts: A Mini Review on their Physicochemical Properties; *J. Chem. Sci. Eng.* **2019**, *2*, 102–104.

[20] Dietz, J., Grote, T., Grammenos, W., Müller, B., Lohmann, J. K., Renner, J., Ulmschneider, S., Vrettou, M., GLÄTTLI, A.; PCT Int. Appl., 2009112533, 17 Sep. 2009.

[21] Seto, Y., Kataoka, M., Tsuge, K., Takaesu, H.; Pitfalls in the toxicological analysis of an isobutyl nitrite-adulterated coffee drink; *Anal Chem.* **2000**, *72*, 5187–5192.

Chapter 3

Analysis of Novichok A-series Degradation Products in Human Urine by Dimethoxytriadinylation LC–MS/MS

3-1. Introduction

Nerve agents are among the most formidable compounds with organophosphorus structures (Figure 3-1A). Because they are classified as chemical weapons, the production, use, and storage of these compounds are strictly controlled under the Chemical Weapons Convention (CWC).[1] However, they are still used in war, terrorist actions, and assasination.[2,3,4,5,6] One of the Novichok A-series compounds, A234 (Figure 3-1A), was used in the attempted assassination of former Russian diplomat Sergei Skripal in 2018;[7,8] its identity was confirmed by the Organization for the Prohibition of Chemical Weapons (OPCW).[9] After this poisoning, Novichok and its related compounds were added to the list of schedule 1 substances of the CWC.[10] These agents were also used in the attempted assassination of Alexei Navalny in 2020.[1111] Thus, these compounds pose an increasing threat to citizens as well as security measures worldwide.

In the event of a chemical terrorist attack, it is necessary to identify the toxic substances responsible. However, they generally cannot be detected in an intact form from environmental or biological samples.[12,13] These compounds are susceptible to hydrolysis to form alkyl methylphosphonic acids (RMPAs) or Novichok degradation products (Figure 3-1A).[14]

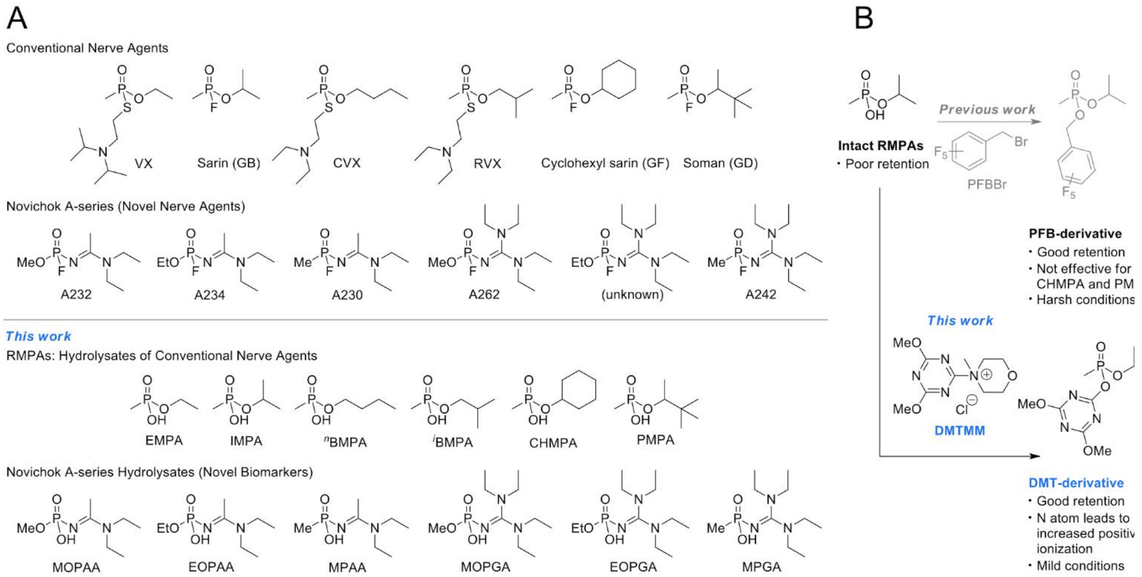


Figure 3-1. (A) Structures of nerve agents and their primary hydrolysis products focused on in this study, (B) conventional methods to detect RMPAs (either intact or after pentafluorobenzylation) and the dimethoxytriadinylation method presented in this paper.

RMPAs have been analyzed by a variety of techniques.[15,16] Liquid chromatography–tandem mass spectrometry (LC–MS/MS) in particular has been widely used recently.[17,18,19] However, the separation of RMPAs from sample matrices is difficult because of their hydrophilicity. In addition, highly hydrophilic compounds such as a VX hydrolysate and Novichok hydrolysates are not retained well by commonly used reversed-phase columns.[13,17,20]

A few methods for the detection of RMPAs in biological samples have achieved sub-ppb detection levels that are sufficient for actual analysis. Although these methods are well designed with a good understanding of the chemical properties of nerve agent hydrolysates, they require solid phase extraction (SPE) or online ZrO_2-columns for concentration and matrix removal.[21,22] In addition, the high polarity of nerve agent hydrolysates necessitates the use of hydrophilic interaction liquid chromatography (HILIC) columns. However, the re-equilibration and reproducibility problems inherent in HILIC columns, as well as the careful removal of large amounts of hydrophilic compounds in biological samples, generally present difficulties.[22]

Conventional derivatization analyses of phosphoric acid and phosphonic acid require harsh conditions because of the insufficient reactivity of derivatizing reagents such as pentafluorobenzyl (PFB) bromide and trimethyl orthoacetate.[23,24,25] Therefore, the byproducts generated by the reaction of the derivatizing reagent with the matrices of the biological samples cause an increase in the background signal even in tandem MS analysis, which deteriorates the limit of detection (LOD) and selectivity of the analysis.[24,25]

In addition, conventional electrophilic derivatization agents (e.g., alkyl halides, acid chlorides, and acid anhydrides) are electronically neutral and hydrophobic, and are strongly retained in reversed-phase columns, causing column clogging and background deterioration.[24,26] Removal of these agents from processed samples is generally laborious or difficult, and even if possible, there is analyte loss due to post-processing steps such as heating, extraction, acidification, basification, or concentration. Therefore, we explored derivatization agents that would not be retained in a reversed-phase column and did not require removal from the samples (Figure 3-1B).

Condensing agents in organic synthesis seemed suitable as derivatizing reagents for LC–ESI(+)/MS because they readily react with acidic functional groups and have nitrogen atoms that are expected to improve positive ionization efficiency. Because most of the hydrolysates of nerve agents are phosphoric acids and phosphonic acids, we assumed that these acidic functional groups would readily react with the condensing agent. However, it was also expected that the activated ester could be readily hydrolyzed back to the original phosphoric or phosphonic acid, so stability would be a key factor in the investigation.

There are few research papers on Novichok, and they are limited to predictions of reactivity and spectra based on theoretical calculations[8,27,28] or reports of the chromatograms and mass spectra of A234.[13] To the best of our knowledge, there is no information on the LOD, quantitative analysis, and screening methods for Novichok. Similarly, there are no reports on the detection of Novichok degradation products in biological samples. One of the reasons for this absence is that these standard compounds are not commercially available. Moreover, the degradation products are electronically charged and difficult to purify using phase separation.

Herein, we report dimethoxytriadinylation LC–MS/MS as a novel method capable of derivatizing phosphoric and phosphonic acids under mild conditions by using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, Figure 3-1B). The degradation products of the Novichok A-series were synthesized in-house without using designated substances. Enabled by the mild and biocompatible dimethoxytriadinylation and the resulting relatively stable activated esters, a simple and sensitive method for the analysis of Novichok A-series and RMPA degradation products using a general-purpose reversed-phase column and LC–MS/MS was achieved. In addition, we report information on LODs, quantitative analysis, and the successful detection of degradation products in urine.

3-2. Experimental

3-2-1. Materials

Ethyl methylphosphonic acid (EMPA) and pinacolyl methylphosphonic acid (PMPA) were purchased from Sigma-Aldrich Co., Inc. (St. Louis, MO, USA). Isopropyl methylphosphonic acid (IMPA), *n*-butyl methylphosphonic acid (^{n}BMPA), isobutyl methylphosphonic acid (^{i}BMPA), cyclohexyl methylphosphonic acid (CHMPA), and the Novichok A-series degradation products *O*-methyl (1-(diethylamino)ethylidene)phosphoramidic acid (MOPAA), *O*-ethyl (1-(diethylamino)ethylidene)phosphoramidic acid (EOPAA), *N*-(1-(diethylamino)ethylidene)-*P*-methylphosphonamidic acid (MPAA), *O*-methyl (bis(diethylamino)methylidene)phosphoramidic acid (MOPGA), *O*-ethyl (bis(diethylamino)methylidene)phosphoramidic acid (EOPGA), and *N*-(bis(diethylamino)methylidene)-*P*-methylphosphonamidic acid (MPGA) were synthesized in our laboratory and were >95% pure by nuclear magnetic resonance (synthetic procedures are shown in the Experimental section). The derivatization reagent, DMTMM (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), was prepared as a 20% (w/v) methanol solution. HPLC-grade solvents were used, and ultra-pure water was purchased from Fujifilm Wako Pure Chemical Co., Ltd. (Osaka, Japan). Drug-free urine (pool, Lee Biosolutions, Inc., Maryland Heights, MO, USA) was used for experiments other than the repeatability test. Drug-free urine from five individual donors (three males and two females, Lee Biosolutions, Inc.) was used for the repeatability test. The other reagents were purchased from Fujifilm Wako Pure Chemical, Kanto Chemical Co., Inc. (Tokyo, Japan), and Tokyo Chemical Industry.

3-2-2. Derivatization reagent and reaction solvent selection

IMPA in a solvent at 0.1 mg/mL or 1 μg/mL (100 μL) was reacted with a 1 mg of condensation agent at 23 °C for 2–6 h, and the yield of derivatized IMPA was determined from the LC–MS analysis.

3-2-3. Sample preparation

Derivatization reactions were conducted in 2 mL microcentrifuge tubes (Safe-Lock Tube, 2.0 mL, colorless, Eppendorf, Hamburg, Germany). A 300 μL of standard solution of nerve agent degradation products or spiked urine (for urine, 16 μL of 10% pyridine in acetonitrile was also added) was pipetted to the tube and dried by centrifugal evaporation at 50 °C to form a pellet. Acetonitrile (100 μL) and 20% DMTMM in MeOH (2 μL) were added to the dried residue. The mixture was incubated at 50 °C for 2 h and then cooled to 23 °C, followed by centrifugation (14,000 × *g*, 5 min at 23 °C) for LC–MS/MS analysis.

3-2-4. LC–MS/MS analysis

The processed sample (3 μL) was injected into a Shimadzu Nexera X2 HPLC system (Shimadzu Corporation, Kyoto, Japan). A 150 mm × 1.5 mm I.D., 5 μm particle size L-column3 ODS column (CERI, Tokyo, Japan) was used for separation at 40 °C. The mobile phase consisted of 0.1% v/v formic acid in water (solvent A) and acetonitrile (solvent B). The gradient mode was 5% B (3 min), increasing to 55% B at 3.3%/min, and then 95% B at 10%/min at a flow rate of 0.2 mL/min. The column effluent was introduced into a Bruker Impact HD Q-TOF mass spectrometer (Bruker, Billerica, MA, USA) via an electrospray ionization mode. Ionization was performed in positive mode. The spray and collision gas were nitrogen. The gas flow rate was 8.0 L/min, the dry temperature was 200 °C, and the ionization spray voltage was 4.5 kV. Acquisition was performed in MRM mode. Precursor ions of protonated molecules ($[M + H]^+$) of DMT-RMPAs and DMT-Novichok A-series degradation products were selected. The transitions obtained, retention times (RTs), and collision energies (CEs) are listed in Table 3-1.

Table 3-1. Optimized LC–MS/MS Acquisition Parameters and Product Ions

Analyte	RT (min)	Precursor ion *m/z* [a]	CE (arbitrary units)	Fragment ions *m/z*
DMT-EMPA	11.4	264.1	25	158.0560 97.0049
DMT-IMPA	12.6	278.1	13	236.0431 158.0560
DMT-^{n}BMPA	14.6	292.1	12	158.0560 236.0431
DMT-^{i}BMPA	14.5	292.1	12	236.0431 158.0560
DMT-CHMPA	15.8	318.1	14	236.0431 158.0560
DMT-PMPA	16.7	320.1	15	236.0431 158.0560
DMT-MOPAA	13.6	348.1	25	150.0678 168.0784
DMT-EOPAA	14.6	362.2	25	164.0835 154.0628
DMT-MPAA	13.3	332.1	22	175.0995 134.0729
DMT-MOPGA	15.0	405.2	22	234.0274 248.1522
DMT-EOPGA	15.8	419.2	22	248.0431 262.1679

[a] Isolation width = ±2 Da.

3-2-5. Extraction efficiency, ion suppression/enhancement, and interference of the derivatization reaction

Concentrated spiked urine (300 µL, 40 ng/mL) was derivatized in 100 µL of acetonitrile. A standard solution of analytes in acetonitrile (100 µL, 120 ng/mL) was added to concentrated blank urine (300 µL) and derivatized. The peak areas obtained from the two samples were compared to calculate the extraction efficiency (*n* = 4).

A solution of derivatized standards in acetonitrile (5 µL, 800 ng/mL) was added to 95 µL of the reaction mixture from the concentration and derivatization of blank urine. A 5 µL sample of the same derivatized standard solution was diluted with 95 μL of acetonitrile. The peak areas obtained from the two samples were compared to calculate ion suppression/enhancement (*n* = 4).

The peak area obtained from the concentrated spiked urine was compared with that of the standard solution in acetonitrile (both 300 μL, 40 ng/mL). The extraction efficiency and ion suppression/enhancement were subtracted from this result to determine the interference of the derivatization reaction by the urine matrices (*n* = 4).

3-2-6. Method Validation

3-2-6-1. Selectivity

Method selectivity was determined by analyzing blank urine samples from five individuals (*n* = 4).

3-2-6-2. Linearity, Sensitivity, Accuracy, and Precision

Calibration curves were prepared from urine samples containing RMPAs or Novichok hydrolysates at concentrations of 0.40, 1.0, 4.0, 10, 40, and 100 ng/mL and analyzed using the derivatization process and LC–MS/MS method described above. The calibration curve for each analyte was the concentration versus the peak area. The LOD was determined to be the concentration at which each selected product ion intensity was approximately 100 counts, and the limit of quantification (LOQ) was determined to be approximately three times the corresponding LOD. The LOD and LOQ were verified by repeatability experiments.

The repeatability of the method was examined using the LOQ concentrations and 40 ng/mL. Spiked urine was divided into five subsamples and analyzed to examine the accuracy (systematic error) and precision (relative standard deviation, RSD) on the same day (intra-day, *n* = 5) and five consecutive days (inter-day, *n* = 5).

3-2-6-3. Stability of derivatized samples

The stability of the derivatized samples in the autosampler was examined using spiked urine samples (40 ng/mL) left at 15 °C for 24 h to confirm the integrity of the analytes in the samples during analysis.

3-2-6-4. Analysis of Urine Samples from the 5th OPCW Biomedical Proficiency Test

The established method was applied to the analysis of two urine samples, U-555 and U-557, from the 5th OPCW Biomedical Proficiency Test. The samples were spiked with ^{n}BMPA (5 ng/mL) and ^{i}BMPA (40 ng/mL), respectively.

3-2-7. Safety Considerations

As DMT-RMPAs and DMT-Novichok A-series degradation products might be toxic at high concentration, all experiments involving activated ester should be performed with protective gloves.

3-3. Results and Discussion

3-3-1. Derivatization reagent selection

An excess of the derivatization reagent is generally required to obtain quantitative results. Accordingly, removal of the excess reagent is a major concern for trace analysis. When derivatizing hydrophilic analytes, hydrophobic reagents such as PFB bromide are usually employed for reversed-phase chromatography.[24] However, the excess reagents are difficult to isolate from the hydrophobic derivatives, and the reaction of derivatizing reagents and matrices in urine, interfering with the detection of trace analytes. To avoid these problems, we focused on using a hydrophilic condensing agent as the derivatization agent.

We first searched for a condensation agent that reacts with IMPA to give an LC–MS-detectable activated ester. IMPA was reacted with the commercially available condensation agents *N,N′*-dicyclohexylcarbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, or DMTMM. The derivative was observed only when DMTMM was used. A key component of these findings was that, contrary to expectations, the activated phosphate and phosphonate esters were stable in acetonitrile during the course of analysis.

3-3-2. Derivatization of IMPA

Next, the solvent for the DMTMM derivatization procedure was optimized. Using IMPA as the substrate, the effects of methanol, 2-propanol, chloroform, ethyl acetate, acetone, and acetonitrile solvents were investigated. The optimal condition used acetonitrile as the solvent.

We then tested whether this dimethoxytriadinylation LC–MS/MS method was compatible with urine. Standard solutions of RMPAs and Novichok hydrolysates in acetonitrile were blended and spiked into urine, and recovery tests were performed. Various extraction conditions, including liquid–liquid extraction, several salting-out assisted liquid–liquid extractions (SALLE),[22,24] and SPE were investigated. For the RMPAs, the extraction was reproducible (EMPA: 62%, other RMPAs: >85%) only when we used SALLE. However, no satisfactory recoveries of the Novichok degradation products, particularly amidine-containing compounds (<2.5%), were achieved using these methods (data not shown). We subsequently shifted out attention to simple concentration of the urine, and successful extraction of the derivatives from the dried urine pellets was made possible by acetonitrile. Fortunately, the dried urine pellet matrix did not dissolve in acetonitrile,[29] and no detectable degradation in instrument performance due to clogging or other problems was observed after repeated analyses. It was also observed that the yield of the derivatization reaction when urine was present varied considerably with the type and amount of base (Table 3-2) owing to the interferences in urine. The type and amount of base were therefore optimized, concentration with 1.6 μL pyridine per 300 μL urine and reaction for 2 h at 50 °C being the optimal conditions.

Table 3-2a. Optimization of the type of base for DMT-derivatization.

(IMPA in urine at 1 μg/mL, 2 h at 23°C, *n* = 3)	Base added to 300μL of urine (μL)					
	0.1	0.2	0.4	0.8	1.6	3.2
Triethylamine	C	C	C	C	C	C
Diisopropylethylamine	C	C	C	C	C	C
N-methylmorpholine	B	B	B	B	B	B
Prydine	B	B	B	A	A	A

A: reproducible, B: not reproducible, C: no signal.

Table 3-2b. Optimization of the amount of pyridine added to urine for the following DMT-derivatization. Area ratio (%) based on (pyridine, temp., time) = (1.6 μL, 50 °C, 2 h).

(IMPA in urine at 1 μg/mL, *n* = 3)	23°C				50°C		
pyridine (μL)	0.5 h	2 h	4 h	6 h	0.5 h	2 h	4 h
0.8	28	53	91	113	55	97	93
1.6	22	62	96	121	63	100	97
3.2	19	51	84	113	51	95	98

Figure 3-2 shows the extracted ion chromatograms (EICs) of a concentrated urine extract where the RMPAs or Novichok A-series hydrolysates were added at 4 ng/mL. The expected structures of fragment ions of DMT-derivatives are shown in Figure 3-3. Peaks corresponding to the derivatized analytes were detected for 11 compounds; MPGA, which had a relatively large steric hindrance, was not detected, suggesting that the reaction of MPGA with DMTMM did not proceed sufficiently under the reaction conditions. Because the reaction at 90 °C (as with the other derivatization reagents[24,25]) decreased the peak area of DMT-RMPAs, we decided not to include MPGA in this simultaneous analysis.

The major advantage of this method is that it requires only commercially available and inexpensive agents, a simple LC–MS/MS instrument, and a C18 column. Most of the reported sensitive methods for the analysis of RMPAs require special ionization methods, unique solid-phase columns, nano liquid chromatography system, a HILIC column, or a combination thereof.[20–22] The present method also has the advantage of requiring only an easy sample preparation and a common instrument for urine while still providing signal intensities by the single TOF mode that showed sensitivity at sub-ppb to ppb. This indicates that generic screening of nerve agent degradation products is possible at least 4 ng/mL.

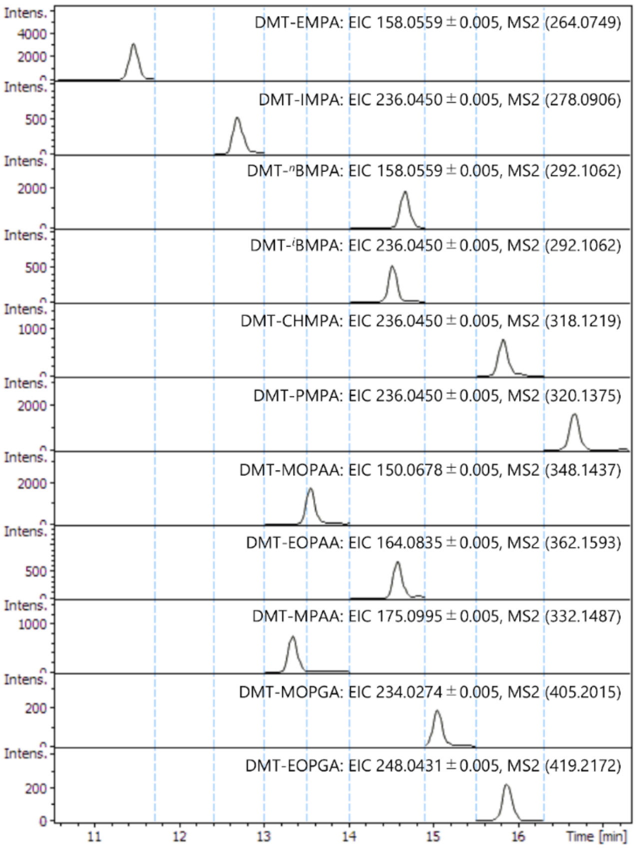


Figure 3-2. EICs from LC–MS/MS of extracts of concentrated urine with added derivatized RMPAs or Novichok A-series degradation products (4 ng/mL).

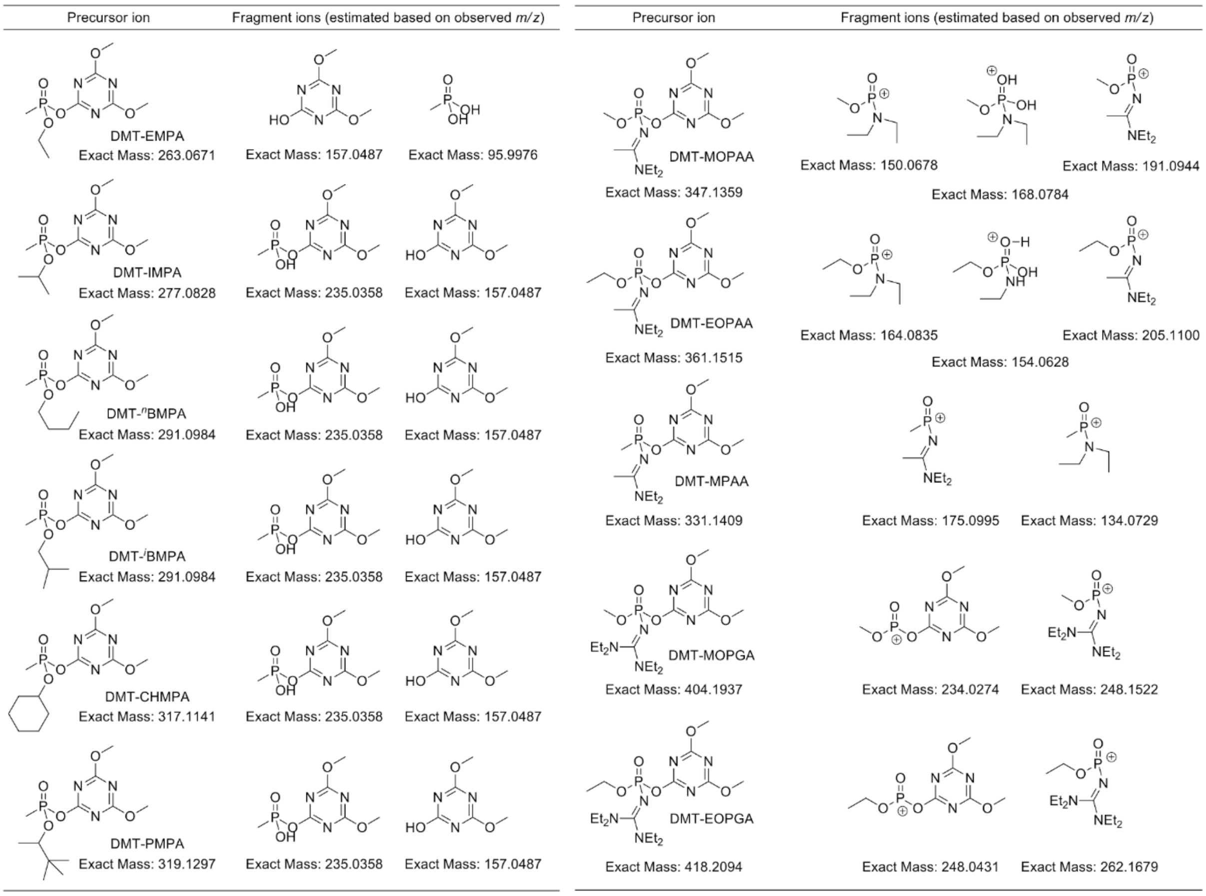


Figure 3-3. Expected structures of fragment ions of DMT-derivatives.

Table 3-3. Method Validation Data for Determination of the Nerve Agent Degradation Products in Urine

	LOD (ng/mL)		LOQ (ng/mL)	Quantitative ion *m/z*	R^2 [b]	Intra-day, accuracy/RSD (%, *n* = 5)		Inter-day, accuracy/RSD (%, *n* = 5)	
	One ion	Two ions				LOQ	40 ng/mL	LOQ	40 ng/mL
EMPA	0.4	4	1	158.0560	0.959	-3.4/19	-2.8/12	-7.3/17	-14/19
IMPA	0.4	1	1	236.0431	0.955	-3.8/18	-12/16	-8.8/22	-11/14
^{n}BMPA	0.4	4	1[a]	292.1062[a]	0.984[a]	4.1/12[a]	11/13[a]	6.9/19[a]	19/11[a]
^{i}BMPA	0.4	1	1	236.0431	0.965	1.8/15	9.0/8.1	-5.0/17	11/14
CHMPA	0.4	0.4	1	236.0431	0.981	-2.0/11	-5.3/6.9	-6.2/18	-17/13
PMPA	0.1	0.4	0.4	236.0431	0.968	-4.3/12	-13/4.1	-10/24	-12/15
MOPAA	0.4	1	4	150.0678	0.963	-6.5/8.3	6.5/7.6	-8.7/14	-16/12
EOPAA	1	1	4	164.0835	0.988	5.3/13	5.4/5.4	7.1/18	10/10
MPAA	1	1	4	175.0995	0.981	-2.5/14	2.1/10	-4.9/18	13/13
MOPGA	4	10	10	234.0274	0.936	16/23	-3.8/15	3.0/20	-13/21
EOPGA	4	10	10	248.0431	0.963	-0.5/20	12/18	8.5/25	10/18

[a] The protonated molecule was used: $[M + H]^+ = 292.1062$, [b] LOQ–100 ng/mL.

3-3-3. Limit of detection

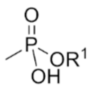
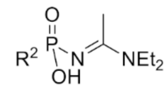
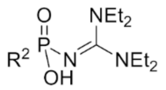
The LOD was determined as the concentration at which the signal intensity of each selected product ion was approximately 100 counts (Table 3-3). This is 3 to 5 times the any noise intensity seen in EICs obtained by MRM mode with a mass accuracy of ± 0.005 Da.

The LODs of RMPAs in urine obtained in this study (0.1–0.4 ng/mL, using one product ion) was slightly inferior to the LOD (<0.24 ng/mL) reported by Mawhinney et al.[21] using a combination of off-line SPE and LC–MS/MS using a silica column, and the LOD (<0.12 ng/mL) reported by Røen et al.[22] using an online SPE-LC–MS/MS system. Recently, a lower LOD (0.02 ng/mL) was also reported for the determination of analytes in urine by GC–MS/MS using PFB derivatization and a highly sensitive negative ion chemical ionization method.[23] However, our method requires only common reagents and less specialized equipment than other methods, and the peak resolution and peak shape are comparable to those of existing methods. Furthermore, the selectivity of the Q-TOF analyzer is superior to that of the triple quadrupole analyzer employed in the reported methods in terms of mass resolution; therefore, the Q-TOF analyzer, which is utilized in our method, is preferable for forensic toxicological analysis.

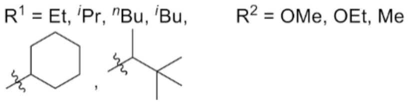
Although several analytical methods utilizing LC–MS/MS for RMPAs in urine have been reported[21,22,24] (Table 3-4), they have several disadvantages: (a) difficulty in extraction[22,24] particularly for amidine-containing compounds, (b) poor reproducibility of SPEs, probably due to the high polarity of the analytes, and (c) severe clogging in the ESI sprayer needle and LC column due to the precipitation of a PFB-related compound. We encountered these problems and failed to consistently reproduce their methods. In contrast, DMTMM is hydrophilic and easily separated from the derivatized analytes by reversed-phase chromatography, which is helpful for avoiding these problems.

To the best of our knowledge, there are no published studies on the concentration of hydrolysis products in urine from the victim exposed to Novichok. However, considering the concentration in urine from exposure to nerve agents such as VX,[3] we can assume that the low ppb measurements achieved with Novichok degradation products in urine is sufficient for practical use.

Table 3-4. Applicability for the Analysis of Degradation Products of Nerve Agents in Urine by LC–MS/MS Methods Reported Previously and in This Paper for Each Type of Degradation Product

Form of product, separation method	Urine pretreatment				Remarks
Underivatized, RP	Extraction[22]	B	C	B	Weak retention
	Concentration	B	C	B	Weak retention
Underivatized, HILIC	Extraction, SPE[22]	A	C	A	C: Not extracted
	SPE[21]		C	C	Not reproducible
PFB, RP	Extraction[24]	B	C	A	C: Not extracted
	Concentration	C	C	C	Line/needle damage
DMT, RP	Extraction[22,24]	A	C	B	B: Not effective for DMT-MPGA
	Concentration	A	A	B	B: Not effective for DMT-MPGA

RP: reversed-phase chromatography, A: suitable, B: partially possible, C: difficult.



3-3-4. Extraction efficiency, ion suppression/enhancement, and interference of derivatization reaction

The extraction efficiency, ion suppression/enhancement, and reaction interference by the matrices are listed in Table 3-5. Almost no ion suppression/enhancement was observed, and the extraction efficiency and interference of the derivatization reaction were approximately 50% and 60%, respectively.

Table 3-5. Breakdown of Yield Losses in Urine Sample Analysis

(*n* = 4)	Extraction efficiency ± SD (%)	Interference of derivatization reaction ± SD (%)	Ion suppression/ enhancement ± SD (%)
EMPA	51 ± 4	52 ± 11	87 ± 5
IMPA	57 ± 6	55 ± 7	94 ± 5
^{n}BMPA	55 ± 4	51 ± 13	99 ± 4
^{i}BMPA	59 ± 4	62 ± 5	99 ± 2
CHMPA	61 ± 3	67 ± 5	100 ± 3
PMPA	65 ± 4	60 ± 6	100 ± 2
MOPAA	48 ± 6	61 ± 5	95 ± 4
EOPAA	53 ± 5	59 ± 6	98 ± 4
MPAA	45 ± 4	54 ± 8	93 ± 3
MOPGA	58 ± 5	56 ± 4	97 ± 2
EOPGA	61 ± 3	61 ± 5	99 ± 3

3-3-5. Method Validation

3-3-5-1. Selectivity

Concentrated blank urine samples from five individuals were derivatized and analyzed in the MRM mode. No significant signal was measured at the retention times of the analytes.

3-3-5-2. Linearity, Sensitivity, Accuracy, and Precision

The developed method was validated using pooled urine samples. Calibration curves (concentration of analyte vs. area value) for the 11 nerve agent degradation products were generated by linear regression analysis using a $1/x^2$ weighting factor (Table 3-6).

Three times the LODs is a generally accepted approximation of LOQs.[24] For simplicity, the LOQs in this study were set as shown in Table 3-3. All calibration curves were linear in the range of the LOQ to 100 ng/mL, and the correlation coefficient R^2 was greater than 0.93.

The intra-day and inter-day accuracy and precision were evaluated using spiked urine samples at the LOQ concentration and 40 ng/mL (Table 3-3). The accuracy values for the intra- and inter-day analyses were less than 13% and 19%, respectively. The RSD values for the intra- and inter-day analyses were less than 23% and 25%, respectively, even at the LOQ concentrations. Considering the frequency of these crisis-management analyses are far from that of routine quality assurance, the errors of the present method are acceptable. We plan to synthesize the corresponding stable isotopes for internal standards to improve accuracy and precision.

Table 3-6.·Regression-line equations, correlation coefficients, and linear ranges for DMT–derivatives.

	Regression-line equation	R^2	Liner range (ng/mL)
EMPA	y = 3515.6 x + 2689.7	0.959	1–100
IMPA	y = 1032.5 x + 312.9	0.955	1–100
^{n}BMPA	y = 3266.6 x + 845.9	0.984	1–100
^{i}BMPA	y = 701.4 x + 923.9	0.965	1–100
CHMPA	y = 1357.6 x + 148.3	0.981	1–100
PMPA	y = 3327.7 x + 1096.5	0.968	0.4–100
MOPAA	y = 384.0 x + 663.7	0.963	4–100
EOPAA	y = 325.2 x + 70.8	0.988	4–100
MPAA	y = 136.9 x - 66.9	0.981	4–100
MOPGA	y = 62.3 x + 76.4	0.936	10–100
EOPGA	y = 85.2 x - 12.4	0.963	10–100

3-3-5-3. Stability of derivatized samples

The stability of the phosphorous/phosphonous-DMT esters in processed urine was investigated at the LOQ concentration and 40 ng/mL. The DMT esters were stable (>80% remaining) for 24 h at 15 °C.

3-3-5-4. Repeatability

Figure 3-4 displays the quantification results for the nerve agent degradation products in the five samples from individuals. At both 40 ng/mL and the LOQ concentrations, the peak area of the sample prepared from individual M2 deteriorated by more than 20% compared with that obtained from the spiked pooled urine. The M2 urine had a distinct cloudiness and strong yellow color, suggesting that this decrease was due to a high amount of matrix.

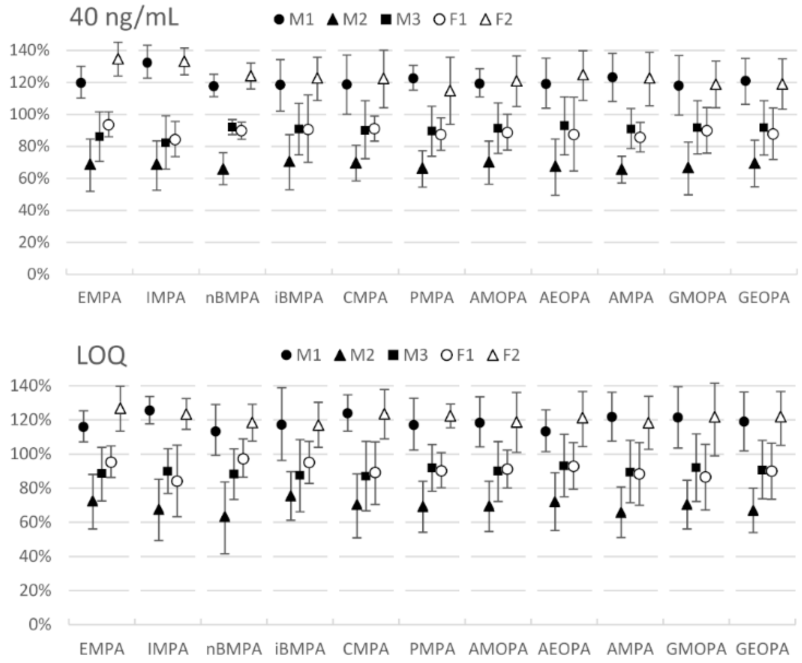


Figure 3-4. Quantification of the nerve agent hydrolysates spiked to five urine samples from individuals at 40 ng/mL and at LOQ concentrations, plotted as mean percent of the true values (based on spiked pooled urine) ± SD (*n* = 4). M = male, F = female.

3-3-5-5. Application of this method

The method was applied to the analysis of two urine samples, U-555 and U-557, collected during the 5th OPCW Biomedical Proficiency Test. Both spiked compounds were successfully identified and quantified (Table 3-7). Note that the nominal concentrations were not always consistent with the true concentrations at the time of analysis.

Table 3-7. Analysis of Urine Samples from the 5th OPCW Biomedical Proficiency Test

Sample	Target compound	Concentration spiked (ng/mL)	Concentration found (ng/mL)
U-555	^{n}BMPA	5	3.3
U-557	^{i}BMPA	40	31

3-4. References

---

[1] Organization for the Prohibition of Chemical Weapons, Chemical Weapon Convention, https://www.opcw.org (Accessed 17 October 2021).

[2] Seto, Y.; Tsunoda, N.; Kataoka, M.; Tsuge, Nagano, T. in: Tu, A. A.; Gaffield, A. (Eds.), Natural and Selected Synthetic Toxins: Biological Imolications, American Chemical Society, Washinton, DC, 1999, pp. 318–332.

[3] Noort, D.; Hulst, A. G.; Platenburg, D. H. J. M.; Polhuijs, M.; Benschop, H. P. Quantitative analysis of O-isopropyl methylphosphonic acid in serum samples of Japanese citizens allegedly exposed to sarin: estimation of internal dosage. *Arch. Toxicol.* **1998**, *72*, 671–675, doi:10.1007/s002040050559.

[4] Public Security Intelligence Agency, https://www.moj.go.jp/psia/25years_after_the_tokyo_subway_sarin_gas_attacks (Accessed 17 October 2021).

[5] Report on the Alleged Use of Chemical Weapons in the Ghouta Area of Damascus on 21 August 2013, http://www.un.org/zh/focus/northafrica/cwinvestigation.pdf (Accessed 17 October 2021).

[6] Jackson, V. *On the Brink: Trump, Kim, and the Threat of Nuclear War*; Cambridge University Press, 2018, 98–99, doi:10.1017/9781108562225.006; ISBN 9781108562225.

[7] Dodd, V.; Harding, L.; MacAskill, E. *Sergei Skripal: former Russian spy poisoned with nerve gas, say police*; The Guardian. https://www.theguardian.com/uk-news/2018/mar/07/russian-spy-police-appeal-for-witnesses-as-cobra-meeting-takes-place (Accessed 17 October 2021).

[8] Bhakhoa, H.; Rhyman, L.; Ramasami, P. Theoretical study of the molecular aspect of the suspected novichok agent A234 of the Skripal poisoning. *R. Soc. open sci.* **2019**, *6*. 181831, doi:10.1098/rsos.181831.

[9] Peplow, M. Nerve agent attack used Novichok poison. *Chem. Eng. News* **2018**, *96*, 3–3.

[10] OPCW, Annex on Chemicals, https://www.opcw.org/chemical-weapons-convention/annexes/annex-chemicals/annex-chemicals (Accessed 17 October 2021).

[11] OPCW, Official Series Document, https://www.opcw.org/sites/default/files/documents/2020/10/s-1906-2020%28e%29.pdf (Accessed 17 October 2021).

[12] Black, R.M.; Read, R. W. Biological markers of exposure to organophosphorus nerve agents. *Arch. Toxicol*. **2013**, *87*, 421–437, doi:10.1007/s00204-012-1005-1.

[13] Lee, J. Y.; Lim, K.C.; Kim, H. S. Characterization and Study on Fragmentation Pathways of a Novel Nerve Agent, 'Novichok (A234)', in Aqueous Solution by Liquid Chromatography-Tandem Mass Spectrometry. *Molecules* **2021**, *26*, 1059, doi: 10.3390/molecules26041059.

---

[14] Munro, N. B.; Talmage, S. S.; Griffin, G. D.; Waters, L. C.; Watson, A. P.; King, J. F.; Hauschild, V. The sources, fate, and toxicity of chemical warfare agent degradation products. *Environ. Health Prospect.* **1999**, *107*, 933–974, doi: 10.1289/ehp.99107933.

[15] Blach, R. M.; Muir, B. Derivatisation reactions in the chromatographic analysis of chemical warfare agents and their degradation products. *J. Cromatogr. A* **2003**, *1000*, 253–281, doi:10.1016/s0021-9673(03)00183-3.

[16] Jang, Y. J.; Kim, K.; Tsay, O. G.; Atwood, D. A.; Churchill, D. G. Update 1 of: Destruction and Detection of Chemical Warfare Agents. *Chem. Rev.* **2015**, *115*, PR1–PR76, doi:10.1021/acs.chemrev.5b00402.

[17] Black, R. M.; Read, R. W. Application of liquid chromatography-atmospheric pressure chemical ionisation mass spectrometry, and tandem mass spectrometry, to the analysis and identification of degradation products of chemical warfare agents. *J. Cromatogr. A* **1997**, *759*, 79–92, doi:10.1016/S0021-9673(96)00763-7.

[18] Hamelin, E. I.; Schulze, N. D.; Shaner, R. L.; Coleman, R. M.; Lawrence, R. J.; Crow, B. S.; Jakubowski, E. M.; Johnson, R. C. Quantitation of five organophosphorus nerve agent metabolites in serum using hydrophilic interaction liquid chromatography and tandem mass spectrometry. *Anal. Bioanal. Chem.* **2014**, *406*, 5195–5202, doi:10.1007/s00216-014-7702-2.

[19] Røen, B. T.; Sellevåg, S. R.; Dybendal, K. E.; Lundanes, E. Trace determination of primary nerve agent degradation products in aqueous soil extracts by on-line solid phase extraction–liquid chromatography–mass spectrometry using ZrO_2 for enrichment. *J. Cromatogr. A* **2014**, *1329*, 90–97, doi:10.1016/j.chroma.2014.01.004.

[20] Lee, J. Y.; Lee, Y. H. Rapid screening and determination of nerve agent metabolites in human urine by LC-MS/MS. *J. Anal. Chem.* **2014**, *69*, 909–916, doi:10.1134/S1061934814090081.

[21] Mawhinney, D. B.; Hamelin, E. I.; Fraser, R.; Sliva, S. S.; Pavlopoulos, A. J.; Kobelski, R. J. The determination of organophosphonate nerve agent metabolites in human urine by hydrophilic interaction liquid chromatography tandem mass spectrometry. *J. Cromatogr. B* **2007**, *852*, 235–243, doi:10.1016/j.jchromb.2007.01.023.

[22] Røen, B. T.; Sellevåg, S. R.; Lundanes, E. Quantification of Nerve Agent Biomarkers in Human Serum and Urine. *Anal. Chem.* **2014**, *86*, 23, 11833–11840, doi:10.1021/ac503408x.

[23] Lin, Y.; Chen, J.; Yan, L.; Guo, L.; Wu, B.; Li, C.; Feng, J.; Liu, Q.; Xie, J. Determination of nerve agent metabolites in human urine by isotope-dilution gas chromatography-tandem mass spectrometry after solid phase supported derivatization. *Anal. Bioanal. Chem.* **2014**, *406*, 5213–5220, doi:10.1007/s00216-014-7695-x.

[24] Otsuka, M.; Tsuge, K.; Seto, Y.; Miyaguchi, H.; Uchiyama, M. Analysis of degradation products of nerve agents via post-pentafluorobenzylation liquid chromatography-tandem mass spectrometry. *J. Cromatogr. A* **2018**, *1577*, 31–37, doi: 10.1016/j.chroma.2018.09.042.

---

[25] Barr, J.R.; Driskell, W. J.; Aston, L. S.; Martinez, R. A. Quantitation of metabolites of the nerve agents sarin, soman, cyclohexylsarin, VX, and Russian VX in human urine using isotope-dilution gas chromatography-tandem mass spectrometry. *J. Anal. Chem.* **2014**, *69,* 909–916.

[26] Maseda, C.; Hayakawa, A.; Okuda, K.; Asari, M.; Tanaka, H.; Yamada, H.; Jin, S.; Horioka, K.; Matoba, K.; Shiono, H.; Matsubara, K.; Shimizu, K. Liquid chromatography-tandem mass spectrometry method for the determination of thiosulfate in human blood and urine as an indicator of hydrogen sulfide poisoning. *Leg. Med.* **2017**, *24*, 67–74, doi:10.1016/j.legalmed.2016.12.004.

[27] Imrit, Y. A.; Bhakhoa, H.; Sergeieva, T.; Danés, S.; Savoo, N.; Elzagheid, M. I.; Rhyman, L.; Andrada, D. M.; Ramasami, P. A theoretical study of the hydrolysis mechanism of A-234; the suspected novichok agent in the Skripal attack. *RSC Adv.* **2020**, *10*, 27884–27893, doi:10.1039/D0RA05086E.

[28] Otsuka, M.; Miyaguchi, H. Theoretical evaluation of the hydrolysis of conventional nerve agents snd novichok agents. *Chem. Phys. Lett.* **2021**, *785*, 139116, doi:10.1016/j.cplett.2021.139116.

[29] Schellinger, A. P.; Carr, P. W. Solubility of Buffers in Aqueous–Organic Effluents for Reversed-Phase Liquid Chromatography. *Lc Gc North America* **June 2004**, *22,* 544–548.

Chapter 4

Paper-Based Analytical Device for the On-site Detection of Nerve Agents

4-1. Introduction

Nerve agents are among the deadliest compounds with an organophosphorus structure. These compounds covalently bind to the serine residue at the active center of acetylcholinesterase (AChE) in cholinergic nerves, inhibiting nerve transmission and causing death by respiratory arrest.[1]

Recently, VX, a non-volatile nerve agent, was used in the 2017 assassination of Kim Jong-nam.[2] Novichok, a non-volatile nerve agent of unknown structure manufactured in the former Soviet Union, was used in the 2018 attempted assassination of former Russian diplomat Sergei Skripal and in the death of a woman using a similar nerve agent.[3] These compounds thus pose a major threat to citizens as well as security measures around the world.

Multiple types of devices have been used to detect these compounds, such as ion mobility spectrometers (IMS),[4] gas detection tubes,[5] portable gas chromatographs,[6] and Raman spectrometers,[7] but most cannot detect non-volatile agents because they use a gas phase for analysis. VX can be detected using a Raman spectrometer if it is a concentrated solution (> 10%), but not if it is a dilute solution. Another problem is the device response to unknown nerve agents. Numerous types of Novichok are believed to have been synthesized by the former Soviet Union,[8] but the structures have not been disclosed, including the one used in the 2018 incident. These agents thus cannot be detected using IMS detectors or Raman spectrometers because the sample data must be collated with a database.

If the detection devices cannot identify non-volatile or unknown nerve agents, first responders (e.g.: riot police and fire department personnel) cannot make accurate judgments on-site, resulting in delays in evacuation orders and life-saving treatment. Therefore, there is an urgent need for an inexpensive device that can quickly and accurately detect nerve agents.

Recent research and development of “lab-on-a-chip” technologies have led to the development of on-site devices for the detection of organophosphorus compounds in aqueous solution,[9,10] in particular, electrochemical microchips and colorimetric biosensors.[11] These approaches have achieved acceptable separation, identification, and limit of detection (LOD) by integrating pretreatment procedures; however, they have several disadvantages, including complex channel design and fabrication, high-voltage manipulation using costly instruments,[12] variable response to organic solvents, temperature, pH and storage as well as dangerous or troublesome stepwise operation.[13]

Microfluidic paper-based analytical devices (µPADs) have been used for point-of-care testing and on-site detection in various analytical fields, including clinical diagnosis and tests for toxins in the context of food and environmental analysis, because a paper substrate has several advantages as an analytical tool.[14,15,16,17,18,19,20,21,22] These advantages are as follows: (1) cellulose-based paper is easily available and cheap; (2) the fibrous cellulose structure can apply capillary force without an external power source and wicks liquids; (3) hydrophobic zones can be formed by typical cutting or printing methods; (4) paper is portable and disposable; and (5) various detection methods can be selected in accordance with each need. Furthermore, colorimetric methods are simple and user-friendly.

These characteristics of μPAD make them suitable for the on-site detection of nerve agents, and there are several research examples of nerve agent detection devices combined with electrochemical sensors[23] and metal complexes.[24] However, no practical examples have been reported to date, likely due to the low stability of the electrode or to the balance between recognizing a wide range of nerve agents and the possibility of false positives due to impurities.

This paper describes a low-cost μPAD for the colorimetric detection of nerve agents in on-site samples using a small sample volume. The key features of this device are that the assay is robust and completed by wicking only. The device configuration is shown in Figure 4-1. After the sample and carrier buffer are placed on the device, three consecutive reactions occur in less than 10 min: inhibition of AChE, hydrolysis of acetylcholine chloride (ACh), and a pH-dependent coloration reaction. The branched sample flow prevents the migration of the free reagent to the edge of the detection window, resulting in a strong and persistent red color resulting from the enzyme inhibition reaction. The developed μPAD had adequate sensitivity (LOD for VX: 2.5 μL of 0.1 μg/mL solution), nerve agent selectivity, reproducibility, robustness, and stability at a low price (at most 20–30 cents). Consequently, the proposed device should provide a practical method for the on-site detection of various non-volatile and unknown nerve agents.

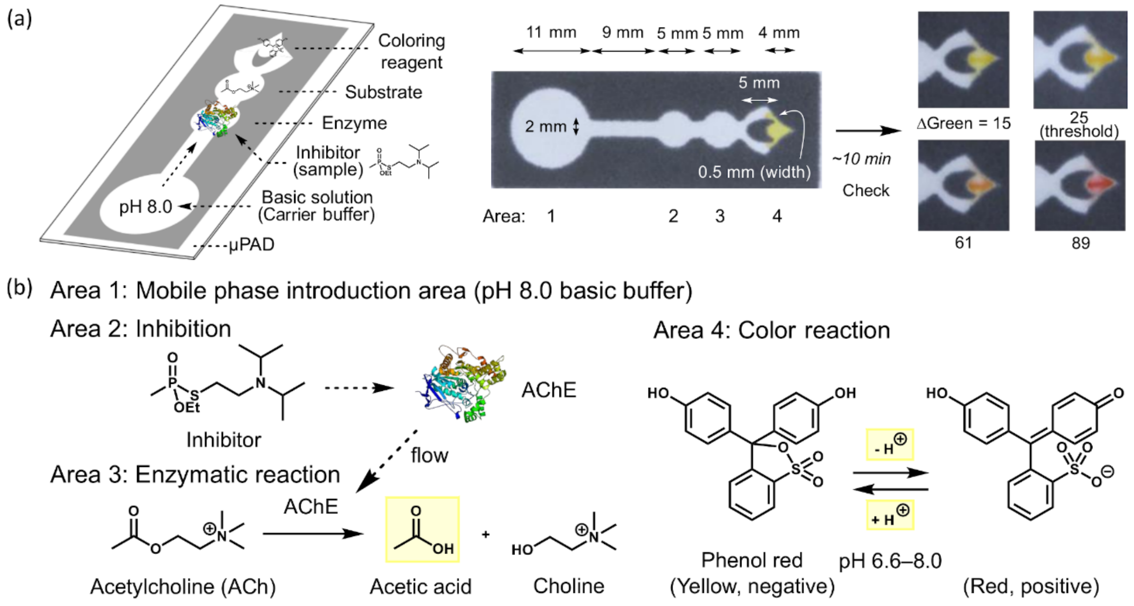


Figure 4-1. (a) Design and photos of the device. (b) Principle for sensing the nerve agent. The sequential reactions occur within approximately 10 min.

4-2. Experimental

4-2-1. Reagents and chemicals

Chromatography paper No. 50 was purchased from Advantec Toyo Kaisha, Ltd. (40×40 cm^2; thickness, 0.25 mm; Tokyo, Japan) and used as the substrate. Standard analyte solutions were prepared prior to analysis using VX, RVX, sarin (GB), and tabun (GA) (Figure 4-2) synthesized in our laboratory (National Research Institute of Police Science; approved by the Ministry of Economy, Trade and Industry, Japan) with great care not to touch the compound directly. Paraoxon and dichlorvos (DDVP) were purchased from Sigma-Aldrich Co., Inc. (St. Louis, MO) and Fujifilm Wako Pure Chemical Co., Ltd. (Osaka, Japan), respectively. Organophosphorus compounds were detected using AChE from *Electrophorus electricus* (electric eel) (Type VI-S, lyophilized powder, 155 or 253 kU/mg (measured by Ellman's assay[25]); Sigma-Aldrich), ACh (Nacalai Tesque, Inc., Kyoto, Japan), and bovine serum albumin (BSA; Sigma-Aldrich), D-(+)-trehalose anhydrous (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), phenol red (Tokyo Chemical Industry), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Nacalai Tesque). Interference was evaluated using drug-free urine (from a single donor, Lee Biosolutions, Inc., Maryland Heights, MO) and common liquid hand-soap (Lion Corporation, Tokyo, Japan). The other reagents were purchased from Fujifilm Wako Pure Chemical, Kanto Chemical Co., Inc. (Tokyo, Japan), or Tokyo Chemical Industry. Aqueous solution samples ([VX] = 0.05−1.0 μg/mL, [RVX] = 0.1−0.5 μg/mL, [GB] = 0.01−0.5 μg/mL, [GA] = 0.1−1.0 μg/mL, [paraoxon] = 20−1000 μg/mL, [DDVP] = 100−5000 μg/mL) were prepared by dilution with 1 mM HEPES-HCl buffer (pH 6.8). All solutions unless otherwise noted were prepared using 1 mM HEPES-HCl buffer (pH 6.8).

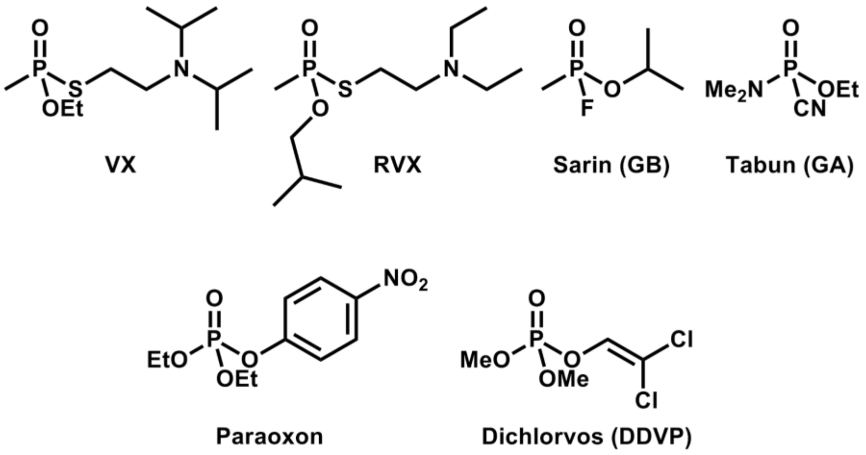


Figure 4-2. Structures of the analytes.

4-2-2. Device fabrication

We used a paper-based device design in which a series of reactions (Figure 4-1) are conducted automatically following sample introduction. The branched flow prevents color-fading. The device comprises a carrier buffer introduction area (area 1), an AChE-inhibition reaction area (area 2), an enzymatic reaction area (area 3) and a color-detection area (area 4). Using this system, the device enables the fast, and reliable detection of nerve agents, even if the agent is non-volatile and/or of unknown structure. The device was designed using Inkscape (v.0.92.1), fabricated using a wax printer (Xerox ColorQube 8570; Xerox Corp., Norwalk, CT), and heated for 4 min at 110°C.

The working principle and the experimental process using the device and a micropipette are shown in Figure 4-1. An aliquot of 1% BSA (2 μL), ACh (1%, 1.5 μL), and phenol red (0.1 w/v%, in 1.0 mM HEPES-HCl buffer (pH 6.8): ethanol (4:1), 1.0 μL) were loaded onto area 2, areas 3, and area 4, respectively, and then dried at room temperature for 15 min. The same drying condition was repeated using trehalose (15 w/w%, 2 μL) and AChE. (0.25 U/μL, 1.75 μL) on area 2.

4-2-3. Device operation and measurement

The sample solution (2.5 μL) was dropped onto area 2 of the device. After approximately 15 seconds, 60 μL of 7.5 mM HEPES-HCl buffer (pH 8.0) (carrier buffer) was added to area 1. After the solution reached the end of the channel, a photograph of area 4 was taken with a digital camera (EOS Kiss X6i; Canon, Tokyo, Japan; distance, ca. 30 cm; ISO, 800; shutter speed, 1/160; aperture value, 5.6) using a light where the green value (in RGB 256 bit) of area 4 before this operation was 128–153. The difference in the green value before and after the operation (ΔGreen = $G_{\text{after}} - G_{\text{before}}$; here denoted red intensity, region of interest (ROI): rectangle, 600 pixels) was obtained by image analysis using GIMP (ver. 2.10.18).

solution. Urine is a typical potentially suspicious material handled by specialized police teams. However, analyzing a victim’s urine is not the purpose of this study.

4-2-5-2. Organic solvents

Samples of 100% organic solvents (ethanol [EtOH], 2-propanol [iPrOH], acetonitrile [MeCN], dimethyl sulfoxide [DMSO] and hexane), and 5% DMSO, were prepared for use as blanks and VX (0.125 μg/mL) was used as the analyte. The influence of each organic solvent on the detection of VX was examined.

4-2-6. Storage test

The tested storage conditions were at room temperature (rt) and 4°C with or without the addition of trehalose (5%, 15%, 34%) under vacuum generated using a vacuum packing machine (Food Saver FM2010, Coleman Japan Co., Ltd. Tokyo, Japan). The blank samples were measured at 6 points (0, 14, 28, 42, 56, and 84 day(s)) after fabrication.

4-2-7. Dilution procedure

We diluted 3 μL VX aqueous solution with 100 μL of the carrier buffer. After mixing with a pipette, 60 μL of the solution was added to the introduction area (area 1) of the device.

4-2-8. Wiping procedure

VX (1–100 ng in acetonitrile), paraoxon (0.4–80 μg in acetonitrile), DDVP (2–160 μg in acetonitrile) was placed on a stainless-steel plate or a plastic plate (acrylonitrile butadiene styrene, ABS) and dried at rt for approximately 20 min. Assuming that on-site samples would be collected with a cotton swab, a piece of cotton (home cotton, compressed by hand to an approx. 5 mm ball; Hakujuji Co. Ltd., Tokyo, Japan) was immersed in 100 μL of the carrier buffer in a microtube and used to wipe one of the surfaces, then the cotton was replaced into the microtube and pressed down. Then 60 μL of the resulting solution was added to area 1.

4-3. Results and Discussion

4-3-1. Optimization of the channel

We examined the flow path using 4 types of detection areas: (tip: sharp or round) × (flow: branched or straight) (Figure 4-3a). The absence of a branch at the end of the flow resulted in color-fading (Figure 4-3b), whereas a branch provided relatively persistent color (Figure 4-3b and d). Moreover, the branched channel, particularly the sharp and branched channel, improved the precision of color development (Figure 4-3e). These promising results are likely due to the end point of the branched flow path being horizontal in the center of the color area and thus retaining the color reagent (Figure 4-3c). In contrast, the straight-type channel results in the carrier buffer gradually permeating the hydrophobic wall from the tip of the flow path, resulting in color-fading. Accordingly, we selected the channel with a sharp tip and a branch.

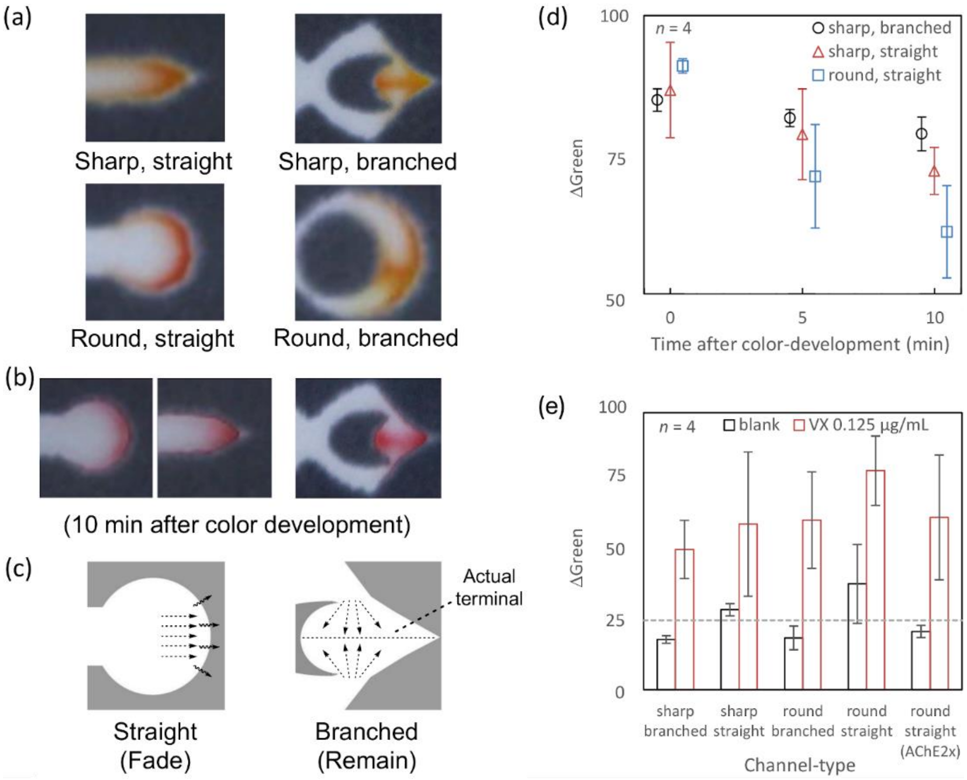


Figure 4-2. Optimization of the detection area. (a) Comparison of the types of coloring areas. A branched path and sharp coloring area is superior to a straight and round area in terms of the visibility of color development. (b) Comparison between a straight path and a branched path, 10 min after color development. (c) Illustration of color fade and persistence. (d) Time-courses of color diffusion. The values of ΔGreen indicate that the rate of red color fading is slower in the branched-type area. (e) ΔGreen = 25: the value that can be confirmed negative visually. The responses for blank and standard sample (VX 0.125 μg/mL) using various channel types. The results show that without branching, the deviation tends to increase for each channel.

4-3-2. Determination of limit of detection

The criterion adopted for determining the LOD was as follows: the concentration at which the value of the 95% confidence interval of ΔGreen should not overlap the value of the positive/negative criterion (ΔGreen = 25).

Figure 4-4 and 5 show the relationship between the concentration of VX, RVX, GB, GA, paraoxon, and DDVP and ΔGreen. The LODs for VX determined by the above criteria were 0.1 μg/mL and 3 μg/mL for AChE 0.44 U and 4.4 U, respectively (Figure 4-5). The LOD for VX is considered to comply with military standards. For instance, the NATO Standard[26] permits exposure to a maximum daily dose of nerve agents of 60 µg.

A point worth noting is that although this device has a high sensitivity to VX, RVX, GB, and GA (nerve agents) the sensitivity to pesticides that inhibit AChE in a manner similar to nerve agents is 1,000 times lower than that of VX (Figure 4-4). This suggests high selectivity for nerve agents in detecting suspicious substances.

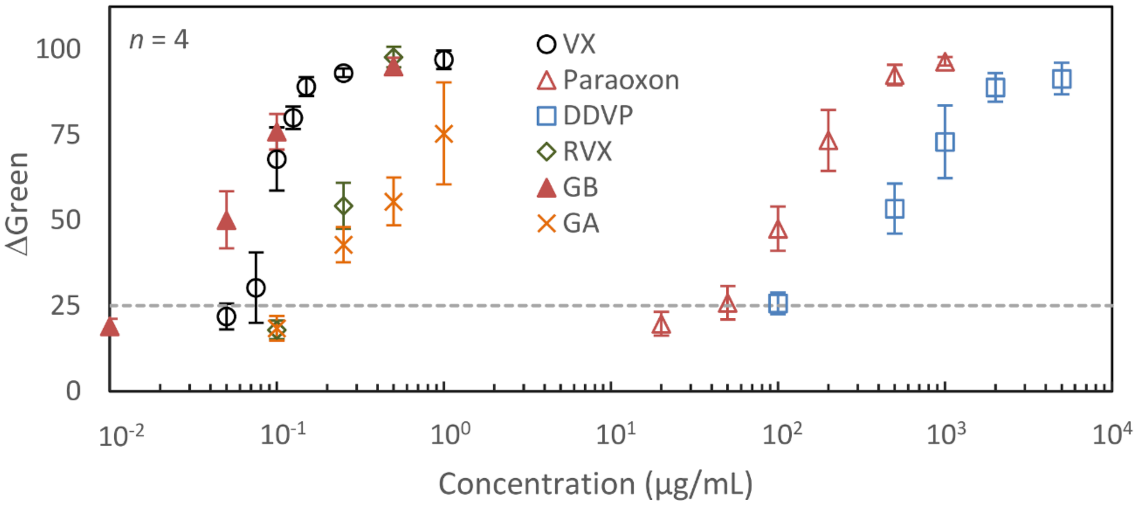


Figure 4-4. VX, RVX, GB, GA, and pesticides responses obtained with the device. Each standard solution was prepared in HEPES-HCl buffer (1.0 mM, pH 6.8). ΔGreen = 25: the value that can be confirmed negative visually. The LOD for VX was determined to be 0.1 µg/mL. (Criteria: The value of the 95% confidence interval of ΔGreen should not overlap the value of the positive/negative criterion (ΔGreen = 25).)

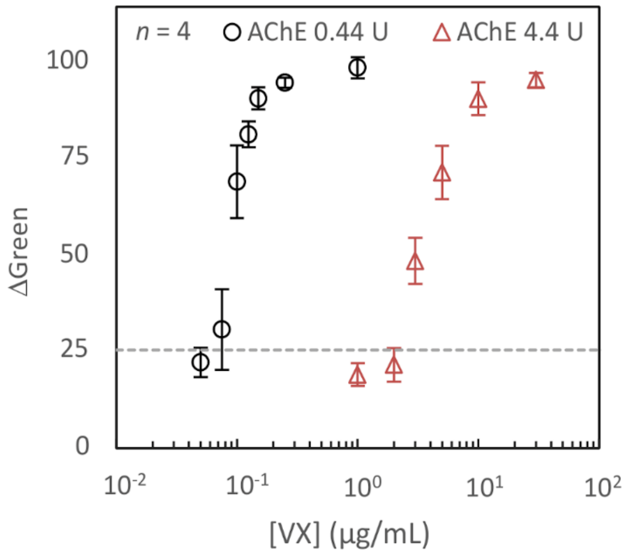


Figure 4-5. Determination of the limit of detection. For 0.44 U and 4.4 U AChE, the LODs for VX determined were 0.1 μg/mL and 3 μg/mL, respectively. (Criteria: The value of the 95% confidence interval of ΔGreen should not overlap the value of the positive/negative criterion (ΔGreen = 25).)

4-3-3. Interference

Figure 4-6a and b indicate that the device should give correct results between pH 4 to 10, unless the pH is buffered with buffer agents (i.e., $NaHCO_3$). We employed a relatively low concentration (7.5 mM) of HEPES-HCl buffer to obtain high sensitivity. Consequently, the final pH of the test sample may be affected by the initial sample pH. This is inevitable when using a pH indicator as the color reagent. This disadvantage can be minimized by diluting the sample prior to its introduction into the device.

The reaction temperature (4°C, 14°C, 33°C; VX (0.125 μg/mL) or blank) did not cause blank samples to give false positives (Figure 4-6c). A decrease in the yield of the reaction of VX with AChE was observed at 4°C, but VX at 0.25 μg/mL correctly gave a positive result. Given that 0.25 μg/mL is sufficiently low to detect VX contamination[26], it was judged that this device is applicable for use in cold conditions.

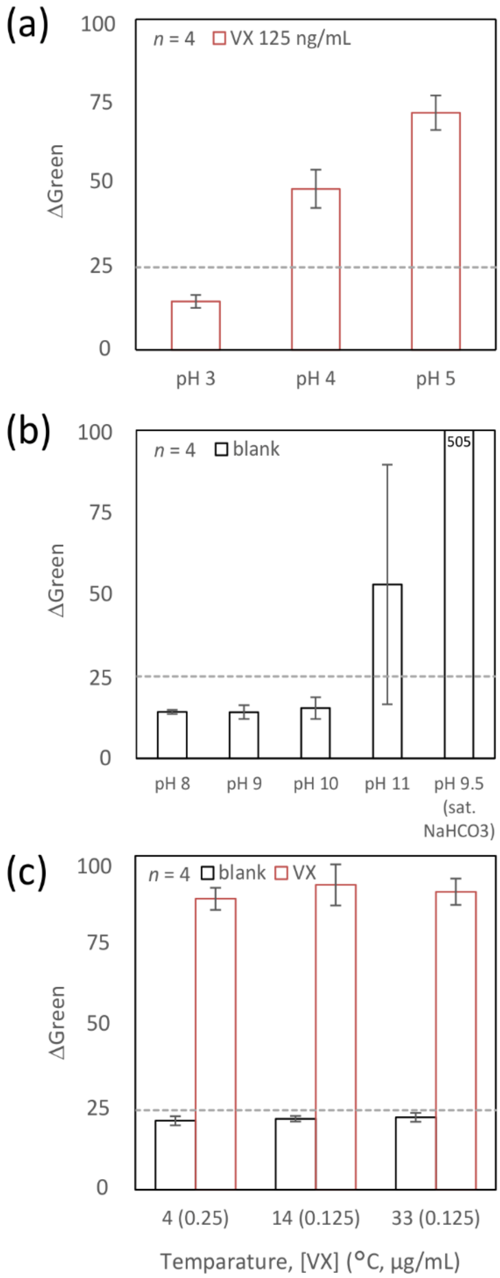


Figure 4-6. Robustness. ΔGreen = 25: the value that can be confirmed negative visually. (a) Evaluation of false negative at low pH. (b) Evaluation of false positive at high pH. The device should give correct results within the pH range 4 to 10. (c) Temperature. The correct result was obtained irrespective of temperature in the range 4°C to 33°C.

Experiments with 100% EtOH, *i*-PrOH, MeCN, hexane and DMSO showed that only 100% DMSO gave a false positive whereas 5% DMSO gave a correct negative (Figure 4-7a). Organic solvents other than DMSO appeared to evaporate rapidly while spreading after their addition, and thus their effect was minimal or zero. In contrast, DMSO did not visibly evaporate and thus likely lead to the observed false positives. When VX was diluted to 0.125 µg/mL in each solvent and added to the device, all samples correctly gave positive results.

Interference by common liquids (coffee, orange juice, urine and hand soap (1% solution)) were examined (Figure 4-7b) and shown not to affect detection. This indicates that typical potentially suspicious material handled by specialized police teams would not lead to false detection results. Considering that the half-life of VX is 1,000 hours (pH 7),[27] it is unlikely that VX becomes undetectable immediately due to hydrolysis in these aqueous samples. However, orange juice slightly decreased the sensitivity of the assay.

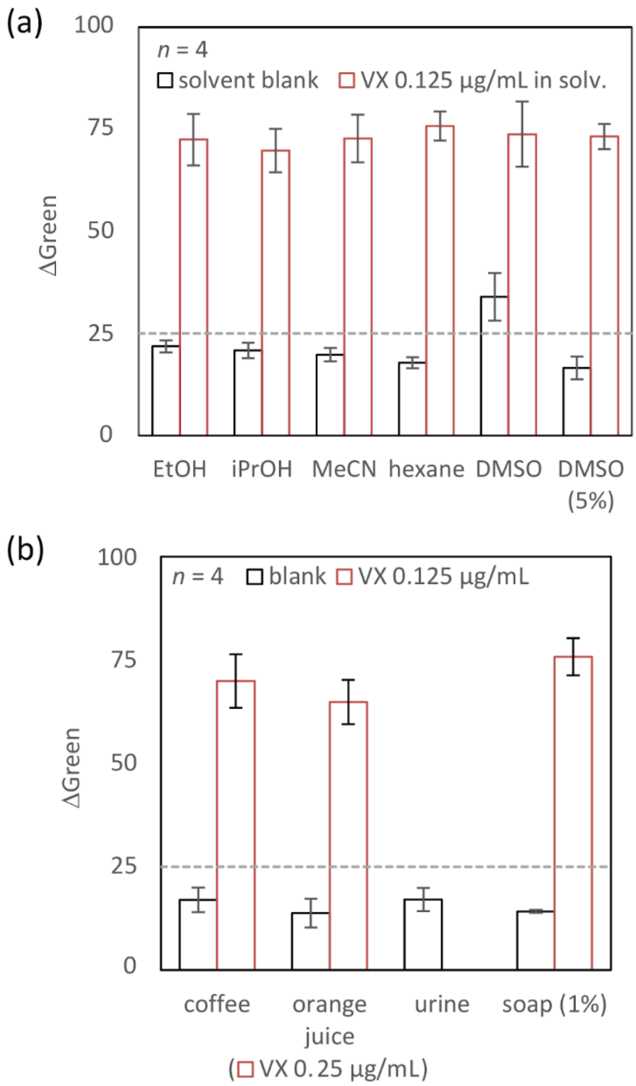


Figure 4-7. (a) Interference due to organic solvents. Only 100% DMSO gave a false result. (b) Interference due to common liquids. These liquids are unlikely to give false results, although orange juice resulted in a slightly lower sensitivity.

4-3-4. Storage stability

Please note that the device provides a false "positive" when the AChE is denatured. The device did not provide a false positive (no overlapping over ΔGreen = 25) in a blank test when the device was fabricated using 0.44 U of AChE with or without trehalose and stored in a vacuum sealer bag at 4°C for 84 days (Figure 4-8). Martinez reported that trehalose improved the stability of enzymes on a paper device.[28] We also observed that trehalose prevented false positives at room temperature 56 days after fabrication (Figure 4-8). Therefore, a 15% trehalose solution was used to improve the stability of AChE.

Although an actual stability test is necessary to determine the shelf-life of the device accurately, here we roughly estimated the shelf-life of the device. Our data revealed that at least 0.264 U of

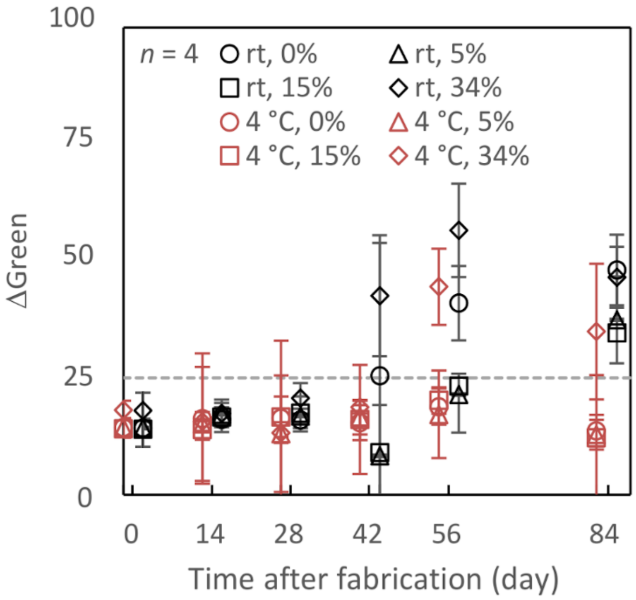


Figure 4-8. Blank test for evaluating the effect of storage temperature and trehalose on shelf-life. The device did not show a false positive (no overlapping over ΔGreen = 25) in a blank test after 84 days if the device was stored under vacuum at 4°C and fabricated using 0%, 5%, or 15 % trehalose.

4-3-5. Pre-dilution and wiping intended for on-site use

As described above, dilution prior to sample introduction is desirable to avoid pH-related issues. We tested an acidic (3 µL of 0.001 mol/L HCl aqueous solution, pH 3, VX 5 ng) and an alkaline (3 µL of 0.001 mol/L NaOH aqueous solution, pH 11, blank) solution, and correct results were obtained by diluting each sample with 100 µL of carrier buffer. Figure 4-9 shows that with pre-dilution or wiping, the LODs were 5–10 ng of VX, which is adequate given the NATO Standard[26] (the maximum daily dose of nerve agents of 60 µg). Thus, dilution prior to sample introduction increased versatility and reliability, making this a practical detection device. Figure 4-10 shows that with wiping, LODs for pesticides were 2 µg of paraoxon and 20 µg of DDVP. The factor contributing to this relatively high LOD is discussed in the next section.

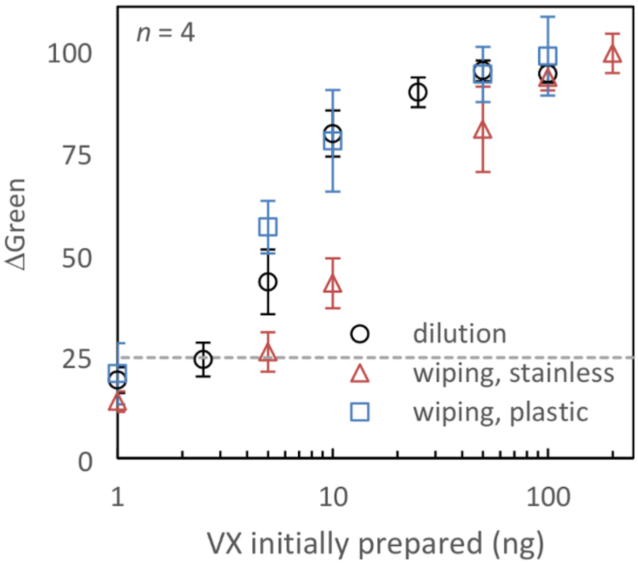


Figure 4-9. Evaluation of LODs for each practical sampling/introduction method. Pre-dilution or wiping provided adequate LODs of 5–10 ng of VX.

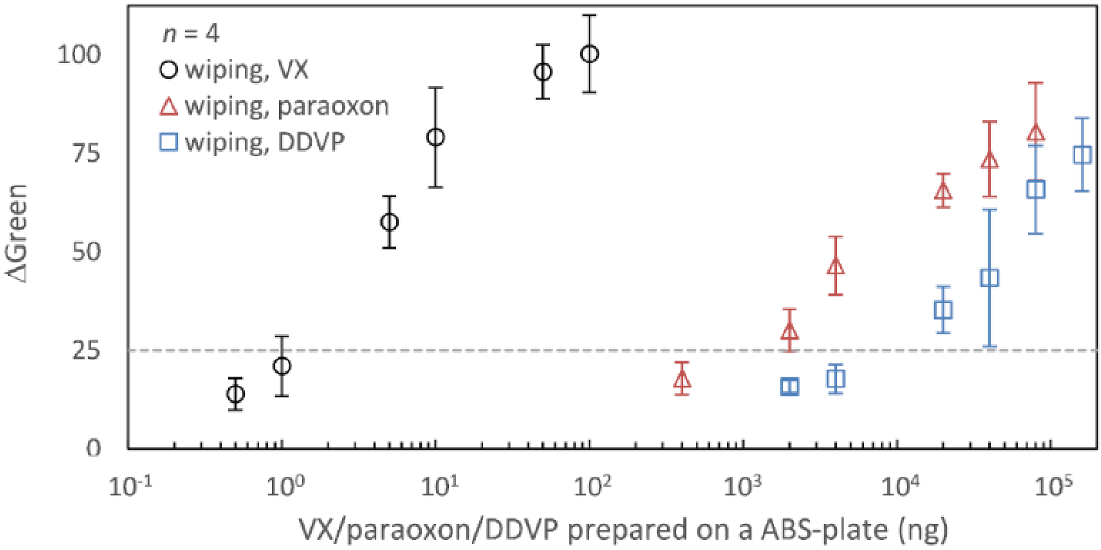


Figure 4-10. VX and pesticide response curve obtained with the device. Each compound was placed on a plastic plate (acrylonitrile butadiene styrene, ABS) and dried at rt for about 20 min and then collected with a piece of cotton (home cotton, compressed by hand to an approx. 5 mm ball) immersed in 100 μL of the carrier buffer (HEPES-HCl buffer (7.5 mM, pH 8.0)). ΔGreen = 25: the value that can be confirmed negative visually.

4-3-6. Comparison of the paper-based device method with a commercial device

A device for the detection of nerve agents reported by Matějovský et al[29] is apparently quite effective and is sold under the brand name “DETEHIT”.[30] This device has an excellent LOD, but little was disclosed regarding its robustness to interference. The device must be immersed in a liquid, then bent and pinched by hand for several minutes, which could be dangerous. Furthermore, the judgement is based only on color which disappears in less than 5–6 min. In contrast, the LOD of our device is inferior, but it has superior robustness, user-friendliness, and color durability.

A comparison of our device with other reported paper devices for pesticide and nerve agent testing and with DETEHIT is shown in Table 4-1[13,31,32,33]. Given the cumbersome procedure, long incubation time, and low robustness of the other devices and the selectivity to nerve agents, the present device is believed to be useful for the rapid testing of unknown materials at the scene of a terrorist attack. The good selectivity of the present device is due in part to the short incubation time, which allows for rapid detection. Pesticides, which have slower reaction rates than nerve agents, result in a lower degree of reaction completeness during a short incubation time.

In comparing the LOD for VX of the present device with DETEHIT, we presume that the low availability of the enzyme due to adsorption on the paper substrate during analysis is one cause of the deterioration in the LOD, leading to an increase in the amount of enzyme to be inhibited. To reduce adsorption due to migration on the substrate, we will explore shortening the flow path by making it three-dimensional, enzyme fixation, and blocking.

Table 4-1. Comparison of organophosphate pesticide and nerve agent detection using various types of paper-based assay devices.

	Mode of optimal detection	Number of assay steps	Sampling method	Assay time (min)	LOD (μg/mL)	LOD (ng)
3D μPAD [31]	Indigo generation	2	Pipetting (200 μL)	50	0.025 (paraoxon)	5
μPAD [32]	Indigo generation	2	Pipetting (6 μL)	35	0.3 (estimated; malathion)	1.8 (estimated)
Paper device [33]	Indigo generation	3	Pipetting (10 μL)	35	0.6 (paraoxon)	6
Dipstick ("Detehit") [13]	Ellman's reagent (redox)	3 (immerse/ wash/fold)	Immersion (bulk)	3–7	0.001 (VX)	- (unknown because of immersion)
Presented μPAD	pH indicator	2	Pipetting (2.5 μL)	8–10	0.1 (VX), 100 (paraoxon)	0.25 (VX), 250 (paraoxon)

	Storage stability	Resistance	Weakness
3D μPAD [31]	> 35 days	-	Assay time (50 min), intricate fabrication
μPAD [32]	> 30 days	-	Assay time (35 min), unclear coloration
Origami-type μPAD 3[3]	3 months	-	Assay time (35 min), unclear coloration
Dipstick ("Detehit") [13]	12 months	Salts (1,000 mg/L), temperature	Narrow pH range (recommended between 6–7), organic solvent (> 30%)
Presented μPAD	> 84 days	pH 4–10, temperature, organic solvent	Relatively high LOD

- : not indicated.

4-4. Conclusion

We here reported a fast, sensitive, and reliable colorimetric method for the detection of nerve agents in on-site samples that does not require operations that could potentially cause erroneous results. The device is practical due to its selectivity to nerve agents, robustness against interference and its stability during storage. The performance of the μPAD was evaluated using nerve agents, demonstrating the true performance of the assay using this μPAD. Although there is still room for improvement in housing and appearance for practical use, and in the detection speed and storage stability, this device provides a practical method for detecting any nerve agent in on-site samples to combat terrorism.

4-5. References

---

[1] Organization for the Prohibition of Chemical Weapons, Chemical Weapon Convention, 2021 (Accessed 16 March 2021) https://www.opcw.org

[2] Jackson, V. *On the Brink: Trump, Kim, and the Threat of Nuclear War*; Cambridge University Press, 2018, 98‑99; DOI:10.1017/9781108562225.006; ISBN 9781108562225

[3] Dodd, V.; Harding, L.; MacAskill, E. *Sergei Skripal: former Russian spy poisoned with nerve gas, say police*; The Guardian. Archived from the original on 7 April 2018. Retrieved 8 March 2018.

[4] Eiceman, G. A; Karpas, Z.; Ion Mobility Spectrometers 2nd ed, Taylor & Francis, Boca Raton, FL, 2005.

[5] Takayama, Y.; Sekioka, R.; Sekiguchi, H.; Maruko, W.; Ohmori, T.; Seto, Y. The Detection Performance of Dräger Safety Gas Detection Tubes for Chemical-Warfare Agents. *Bunseki Kagaku* **2007**, *56*, 355–362.

[6] Henry, C. Taking the Show on the Road: Portable GC and GC/MS. *Anal. Chem.* **1997**, *69*, 195A–201A.

[7] Satoh, T.; Sekioka, R.; Seto, Y.; Matsumiya, H.; Nakagawa, H.; Analysis of Chemical Warfare Agents by Portable Raman Spectrometer. *Bunseki Kagaku*, **2016**, *65*, 539–545.

[8] Chai, R. P.; Hayes, D. B.; Erickson, B. T.; Boyer, W. E. Novichok Agents: a Historical, Current, and Toxicological Perspective. *Toxicol. Commun.* **2018**, *2*, 45–48, doi: 10.1080/24734306.2018.1475151

[9] Diauudin, F. N.; Rashid, J. I. A.; Knight, V. F.; Yunus, W. M. Z. W.; Ong, K. K.; Kasim, N. A. M.; Halim, N. A.; Noor, S. A. M. A Review of Current Advances in the Detection of Organophosphorus Chemical Warfare Agents Based Biosensor Approaches. *Sens. Bio-Sens. Res.* **2019**, 26, 100305, doi: 10.1016/j.sbsr.2019.100305

[10] Pohanka, M.; Karasova, J. Z.; Kuca, K.; Pikula, J.; Holas, O.; Korabecny, J.; Cabal, J. Colorimetric Dipstick for Assay of Organophosphate Pesticides and Nerve Agents Represented by Paraoxon, Sarin and VX. *Talanta* **2010**, *81*, 621-624, doi: 10.1016/j.talanta.2009.12.052

[11] Dhull, V.; Gahlaut, A.; Dilbaghi, N.; Hooda, V. Acetylcholinesterase Biosensors for Electrochemical Detection of Organophosphorus Compounds: A Review. *Biochem. Res. Int*. **2013**, 731501, doi: 10.1155/2013/731501

[12] Liu, G.; Wang, J.; Barry, R.; Petersen, C.; Timchalk, C.; Gassman, P.; Lin, Y. Nanoparticle-Based Electrochemical Immunosensor for the Detection of Phosphorylated Acetylcholinesterase: An Exposure Biomarker of Organophosphate Pesticides and Nerve Agents. *Chem. Eur. J.* **2008**, *14*, 9951–9959, doi: 10.1002/chem.200800412

[13] Pitschmann, V.; Matějovský, L.; Lobotka, M.; Dědič, J.; Urban, M.; Dymák, M. Modified Biosensor for Cholinesterase Inhibitors with Guinea Green B as the Color Indicator. *Biosensors*

2018, *8*, 81, doi: 10.3390/bios8030081

[14] Wu, M.; Lai, Q.; Ju, Q.; Li, L.; Yu, H. D.; Huang, W. Paper-Based Fluorogenic Devices for In Vitro Diagnostics. *Biosens. Bioelectron.* **2018**, *102*, 256–266, doi: 10.1016/j.bios.2017.11.006

[15] Sandbhor Gaikwad, P.; Banerjee, R. Advances in Point-of-Care Diagnostic Devices in Cancers. *Analyst* **2018**, *143*, 1326–1348, doi: 10.1039/C7AN01771E

[16] Sher, M.; Zhuang, R.; Demirci, U.; Asghar, W. Paper-Based Analytical Devices for Clinical Diagnosis: Recent Advances in the Fabrication Techniques and Sensing Mechanisms. *Expert Rev. Mol. Diagn.* **2017**, *17*, 351–366, doi: 10.1080/14737159.2017.1285228

[17] Mahato, K.; Srivastava, A.; Chandra, P. Paper Based Diagnostics for Personalized Health Care: Emerging Technologies and Commercial Aspects. *Biosens. Bioelectron.* **2017**, *96*, 246–259, doi: 10.1016/j.bios.2017.05.001

[18] Almeida, M. I. G. S.; Jayawardane, B. M.; Kolev, S. D.; McKelvie, I. D. Developments of Microfluidic Paper-Based Analytical Devices (μPADs) for Water Analysis: A Review. *Talanta* **2018**, *177*, 176–190, doi: 10.1016/j.talanta.2017.08.072

[19] Lin, Y.; Gritsenko, D.; Feng, S.; Teh, Y. C.; Lu, X.; Xu, J. Detection of Heavy Metal by Paper-Based Microfluidics. *Biosens. Bioelectron.* **2016**, *83*, 256–266, doi: 10.1016/j.bios.2016.04.061

[20] Meredith, N. A.; Quinn, C.; Cate, D. M.; Reilly, T. H.; Volckens, J.; Henry, C. S. Paper-Based Analytical Devices for Environmental Analysis. *Analyst* **2016**, *141*, 1874–1887, doi: 10.1039/C5AN02572A

[21] Busa, L. S. A.; Mohammadi, S.; Maeki, M.; Ishida, A.; Tani, H.; Tokeshi, M. Advances in Microfluidic Paper-Based Analytical Devices for Food and Water Analysis. *Micromachines* **2016**, *7*, 86, doi: 10.3390/mi7050086

[22] Komatsu, T.; Maeki, M.; Ishida, A.; Tani, H.; Tokeshi, M. Paper-Based Device for the Facile Colorimetric Determination of Lithium Ions in Human Whole Blood. *ACS Sensors* **2020**, *5*, 1287–1294, doi: 10.1021/acssensors.9b02218

[23] Cinti, S.; Minotti, C.; Moscone, D.; Palleschi, G.; Arduini, F. Fully Integrated Ready-to-Use Paper-Based Electrochemical Biosensor to Detect Nerve Agents. *Biosens Bioelectron.* **2017**, *93*, 46–51, doi: 10.1016/j.bios.2016.10.091

[24] Martí, A.; Costero, A. M.; Gaviña, P.; Parra, M. Triarylcarbinol Functionalized Gold Nanoparticles for the Colorimetric Detection of Nerve Agent Simulants. *Tetrahedron Lett.* **2014**, *55*, 3093–3096, doi: 10.1016/j.tetlet.2014.03.139

[25] Kostelnik, A.; Cegan, A.; Pohanka, M. Acetylcholinesterase Inhibitors Assay Using Colorimetric pH Sensitive Strips and Image Analysis by a Smartphone. *Int. J. Anal. Chem.* **2017**, 371284, doi: 10.1155/2017/3712384

[26] *Requirements for Water Potability during Field Operations and in Emergency Situations*;

Version 1; NATO Standardization Agency: Brussels, Belgium, 2013.

[27] Munro, N. B.; Talmage, S. S.; Griffin, G. D.; Waters, L. C.; Watson, A. P.; King, J. F.; Hauschild, V. The Sources, Fate, and Toxicity of Chemical Warfare Agent Degradation Products. *Environ. Health Perspect.***1999**, *107*, 933–947, doi: 10.1289/ehp.99107933

[28] Ganaja, K. A.; Chaplan, C. A.; Zhang, J.; Martinez, N. W.; Martinez, A. W. Paper Microzone Plates as Analytical Tools for Studying Enzyme Stability: A Case Study on the Stabilization of Horseradish Peroxidase Using Trehalose and SU-8 Epoxy Novolac Resin. *Anal. Chem.* **2017**, *89*, 10, 5333–5341, doi: 10.1021/acs.analchem.6b05111

[29] Matějovský, L.; Pitschmann, V. New Carrier Made from Glass Nanofibres for the Colorimetric Biosensor of Cholinesterase Inhibitors. *Biosensors* **2018**, *8*, 51, doi: 10.3390/bios8020051

[30] Tušarová, I.; Halámek, E. Biosenzor pro Detekci a Rozlišení Inhibitorů Cholinesteráz. C.Z. Patent 288 576, 22 May 2001.

[31] Hua Q. T.; Shibata, H.; Hiruta, Y.; Citterio, D.Flow Control-Based 3D µPADs for Organophosphate Pesticide Detection. *Anal. Sci.* **2019**, *35*, 393–399, doi: 10.2116/analsci.18P435

[32] Murase, R.; Kondo, S.; Kitamura, T.; Goi, Y.; Hashimoto, M.; e, Y. Cellulose Nanofibers as a Module for Paper-Based Microfluidic Analytical Devices: Labile Substance Storage, Processability, and Reaction Field Provision and Control. *ACS Appl. Bio Mater.* **2018**, *1*, 480–486, doi: 10.1021/acsabm.8b00206

[33] Apilux, A.; Isarankura-Na-Ayudhya, C.; Tantimongcolwat, T.; Prachayasittikul, V. Paper-Based Acetylcholinesterase Inhibition Assay Combining a Wet System for Organophosphate and Carbamate Pesticides Detection. *EXCLI J.* **2015**, *14*, 307–319, doi: 10.17179/excli2014-684

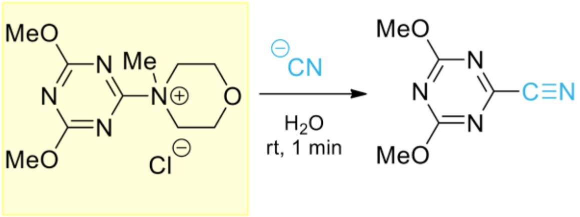
Chapter 5

Conclusion

5-1. A screening method for cyanide in blood by dimethoxytriazinyl derivatization–GC/MS

A simple screening analysis of cyanide in blood has been developed, using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM). DMTMM, a convenient reagent for dehydrocondensation, converted cyanide to 2-cyano-4,6-dimethoxy-1,3,5-triazine, the dimethoxytriazinyl derivative of cyanide. This reaction proceeded in whole blood samples after treatment with trichloroacetic acid, and in basic aqueous solution samples. Sufficient sensitivity was observed by the method using gas chromatography/mass spectrometry. Intra- and inter-day repeated analyses (0.05, 0.1, 0.25, 1, and 5 µg/mL, $n = 5$) were performed and the accuracy and precision were within 20% for the lower limit of quantification (LLOQ) and within 15% for other concentrations. LLOQs for the aqueous solution and blood were 0.05 and 0.1 µg/mL, respectively, which are suitable for detecting cyanide poisoning. The limits of detection (signal-to-noise ratio ≥3) for aqueous solution and blood were 0.01 and 0.05 µg/mL, respectively. Interference from 13 other anions was tested and no false positive response was obtained, even in the case of thiocyanate, nitrite and nitrate, which are known to yield cyanide by acid treatment of blood.

This method is practical because it uses readily available reagents and equipment and is sensitive enough for the rapid screening of cyanide poisoning in forensic and clinical toxicology. In addition, the method provides a simple, low-toxicity derivatization reaction with sufficient intra- and inter-day quantification and screening properties, which is difficult to conduct using other reagents. The aqueous one-phase system reaction we present may be useful for derivatizing other anionic toxicants (e.g., azide, sulfide, and thiosulfate). Because of the simplicity of the reagent system and the mildness of the conditions, this reaction may provide an analytical method for highly water-soluble anions.



- A simple, mild, and low-toxicity derivatization reaction
- Sufficient intra- and inter-day quantification and screening properties
- No false positive/negative for other anions
- Only 5 min-pretreatment for blood sample

Figure 5-1. A screening method for quantifying cyanide in aqueous solution and in biological samples by using the condensation reagent DMTMM.

5-2. Development of DMT derivatization–LC–MS/MS method for of nerve agent hydrolysates

A-series degradation products (hydrolysates of A230, A232, A234, A262, and one related compound) and alkyl methylphosphonic acids (RMPAs, conventional nerve agent hydrolysates) using liquid chromatography–tandem mass spectrometry (LC–MS/MS) was developed. The dimethoxytriadinyl (DMT)-activated esters of Novichok A-series degradation products were found to be stable and readily detected. We used this derivatization to achieve the first analytical method for Novichok hydrolysis products in urine (0.40–4.0 ng/mL). The detection limits of the RMPAs (0.1–0.4 ng/mL) were comparable to those of previous reports involving pentafluorobenzylation or direct LC–MS/MS. The applicability of the newly developed method was evaluated by analyzing urine samples from the OPCW 5th Biomedical Proficiency Test.

To the best of our knowledge, this is the first method intended for the screening of Novichok A-series hydrolysates. The method was also found to be comparable or superior in many ways to conventional intact analysis and pentafluorobenzyl derivatization analysis. In particular, the sensitive and practical analysis of Novichok A-series degradation products in biological samples was very difficult to achieve by other methods reported before 2021. Improvement of RSD and development of a method that can simultaneously detect bulky compounds (e.g. MPGA) are future challenges. We believe that DMT derivatization can be used to expand the range of compounds analyzed, owing to its characteristic properties suitable for hydrophilic analytes. The present method can be implemented using commercially available, inexpensive reagents, general-purpose reversed-phase columns, and common LC–MS equipment, making it easy to implement for many laboratories with limited resources. These features are especially important for the analysis of these terrorism-related compounds because the opportunity for the analysis is expected to be rare but should be prepared for from the standpoint of public safety. Finally, considering the growing interest in Novichok as a new threat to citizens and as a security measure, we hope that the microsynthesis method of Novichok hydrolysate will be useful for the synthesis of reference materials in various laboratories dealing with chemical terrorism cases.

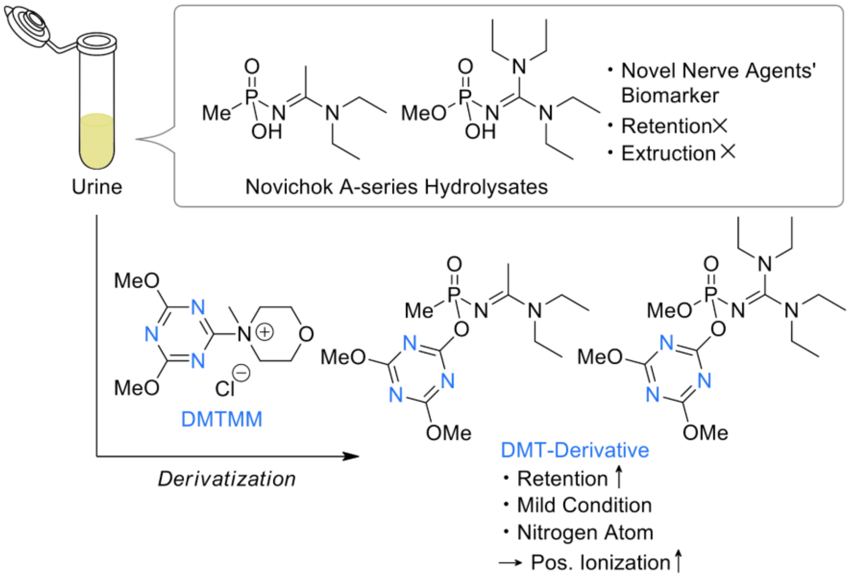


Figure 5-2. A screening method for nerve agents’ hydrolysates in urine by using DMTMM.

5-3. Paper-based analytical device for the on-site detection of nerve agents

We report a colorimetric paper-based microfluidic device based on an enzyme-inhibition assay that allows the on-site detection of nerve agents by sampling and wicking. The sample and reagents are automatically transported through the channel where an enzyme inhibition reaction is conducted, followed by an enzyme-substrate reaction and a color reaction. This device can detect 0.1 µg/mL of the nerve agent VX in a 2.5-µL drop and is nerve agent selective, robust against temperature, pH, and several liquids. We confirmed that sampling procedures (dilution, wiping) are applicable to this device. Furthermore, the fabrication procedure is easy and the cost is at most a few tens of cents. Thus, the present device provides a practical method for the urgent detection of nerve agents in suspected chemical terrorism incidents. The device is practical due to its selectivity to nerve agents, robustness against interference and its stability during storage. The performance of the µPAD was evaluated using nerve agents, demonstrating the true performance of the assay using this µPAD. Although there is still room for improvement in housing and appearance for practical use, and in the detection speed and storage stability, this device provides a practical method for detecting any nerve agent in on-site samples to combat terrorism.

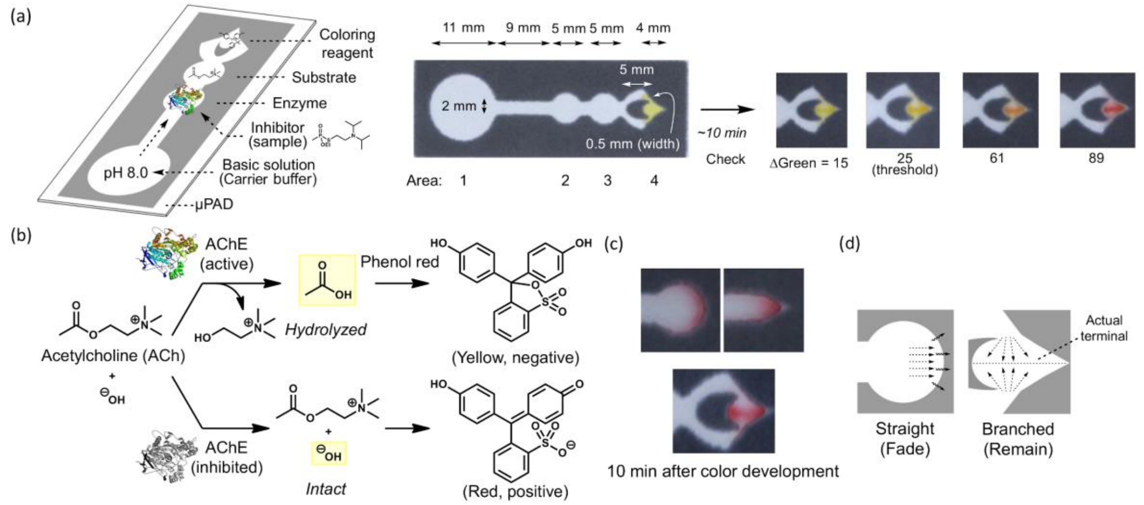


Figure 5-3. (a) Design and photos of the device. (b) Principle for sensing the nerve agent. After the sample and carrier buffer are placed on the device, three consecutive reactions occur in less than 10 min: inhibition of AChE, hydrolysis of acetylcholine chloride (ACh), and a pH-dependent coloration reaction. (c) At the end of the channel (Area 4), the branched flow prevents the migration of the free reagent to the edge of the detection window providing a strong and persistent color development resulting from the enzyme inhibition reaction. (d) Illustration of color fade and persistence.

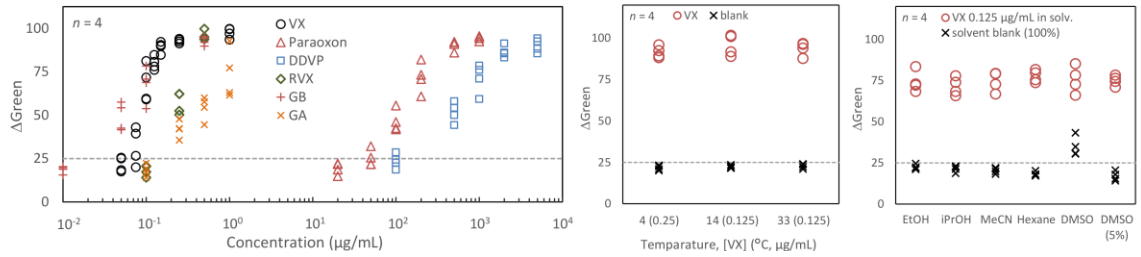


Figure 5-4. (a) VX, RVX, GB, GA, and pesticides responses obtained with the device. The LODs for VX, paraoxon, and DDVP determined according to the above criteria were 0.1 μg/mL, 100 μg/mL, and 500 μg/mL, respectively. The LOD for VX is considered to comply with military standards. For instance, NATO Standard permits the maximum daily dose of nerve agents of 60 μg [1]. Although this device has high sensitivity to nerve agents, the sensitivity to pesticides that inhibit AChE like nerve agents is 1000 times lower than that of VX. This suggests a high selectivity for nerve agents in detecting suspicious substances. (b) Robustness against Temperature, (c) Interference due to organic solvents.

5-4. Future perspective

In this thesis, we report the results of our research on compounds whose performance is insufficient or impossible to be analyzed by the existing analytical methods, using either the newly discovered reaction of DMTMM or the newly designed paper fluid analysis device, both of which are the first steps for future development.

The usefulness of DMTMM for the derivatization of cyanide ions and nerve agent degradation products over conventional derivatization methods suggests the potential for the development of DMTMM and triazine skeletons as derivatization reagents. Specifically, we believe that DMTMM and the triazine skeleton can be applied to the analysis of various high-polarity compounds that are not suitable for conventional LC–MS analysis because they exist as anions in aqueous solution. These high-polarity compounds include chemical agents including cyanide, azide, hydrogen sulfide, thiosulfate, pesticides, and nerve agent degradation products that are problematic in terrorism, crime, and incidents. I believe that the high reactivity of DMTMM and its robustness to matrices will enable us to develop a method to screen more toxic compounds with the same pretreatment method and the same general-purpose LC–MS system.

As for the paper microfluidic analytical device for on-site detection of nerve agents used in terrorism, this time only the detection device of nerve agents was available, but we believe that it is possible to develop a paper chip for simultaneous analysis of all kinds of chemical agents, which is currently possible only with expensive on-site detection devices, by branching off to a flow path with the detection function of other chemical agents introduced in Chapter 1. For nerve agents, which are particularly important to detect, we need to develop a device that can detect them in a shorter time by reconstructing the flow path structure and understanding the reaction efficiency on paper. In addition, we believe that it will be possible to further improve not only counter-terrorism but also illegal drug arrests by developing a simultaneous detection device for use in arresting people in possession of illegal drugs using the knowledge of the device structure obtained in these studies.

The development of such a method should make police investigations, rescue in the site, and the preparation of evidence for trial more rapid, accurate, and robust.

Experimental Section

Chapter 2: Spectral data

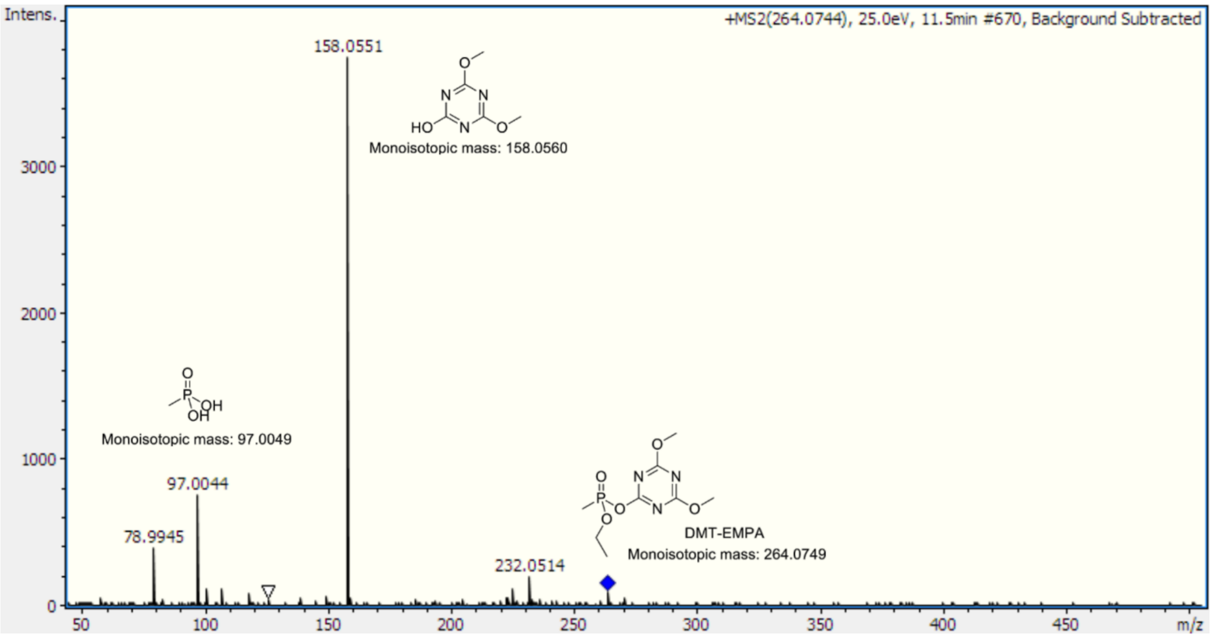


Figure S1. Product ion spectrum of DMT-EMPA.

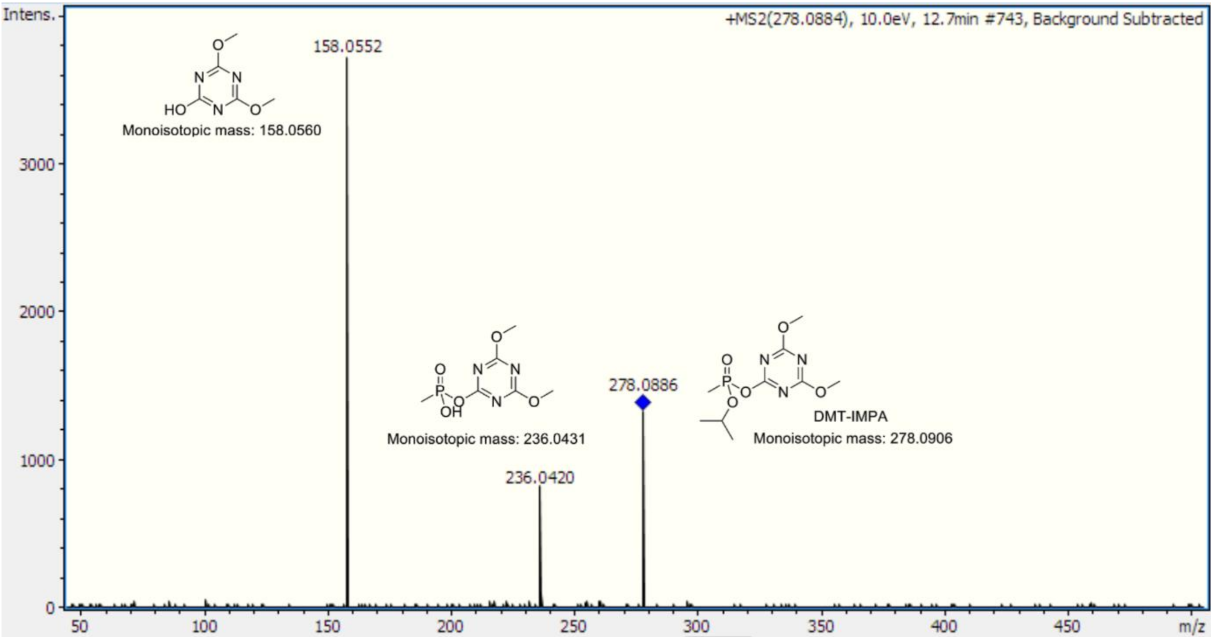


Figure S2. Product ion spectrum of DMT-IMPA.

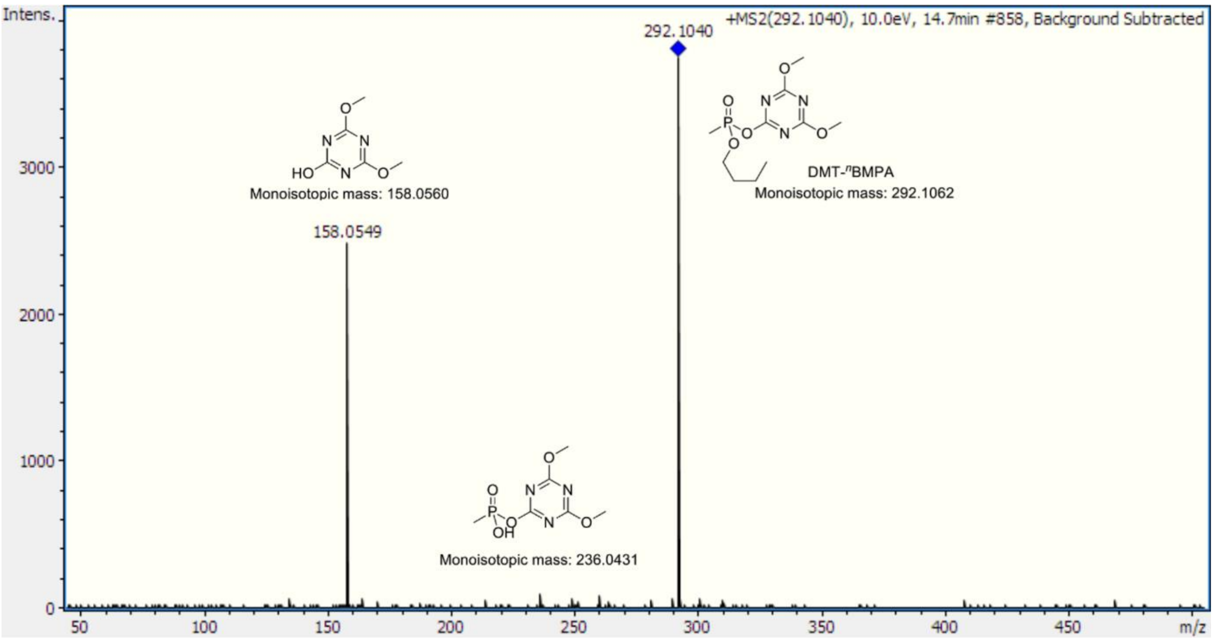


Figure S3. Product ion spectrum of DMT-^{n}BMPA.

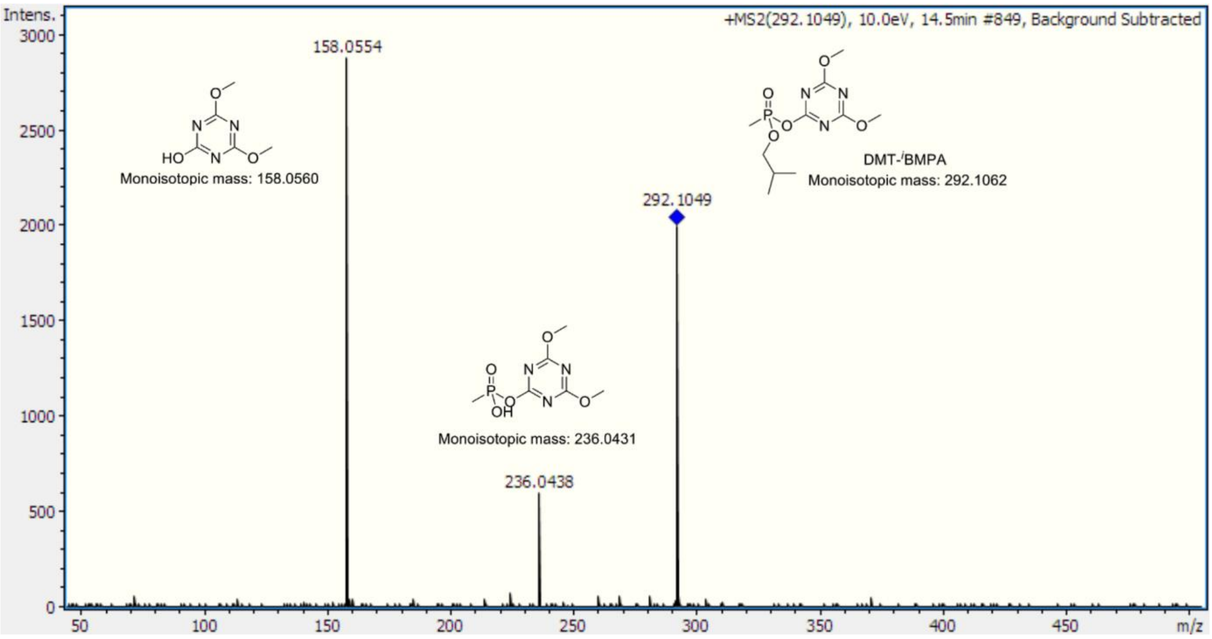


Figure S4. Product ion spectrum of DMT-^{i}BMPA.

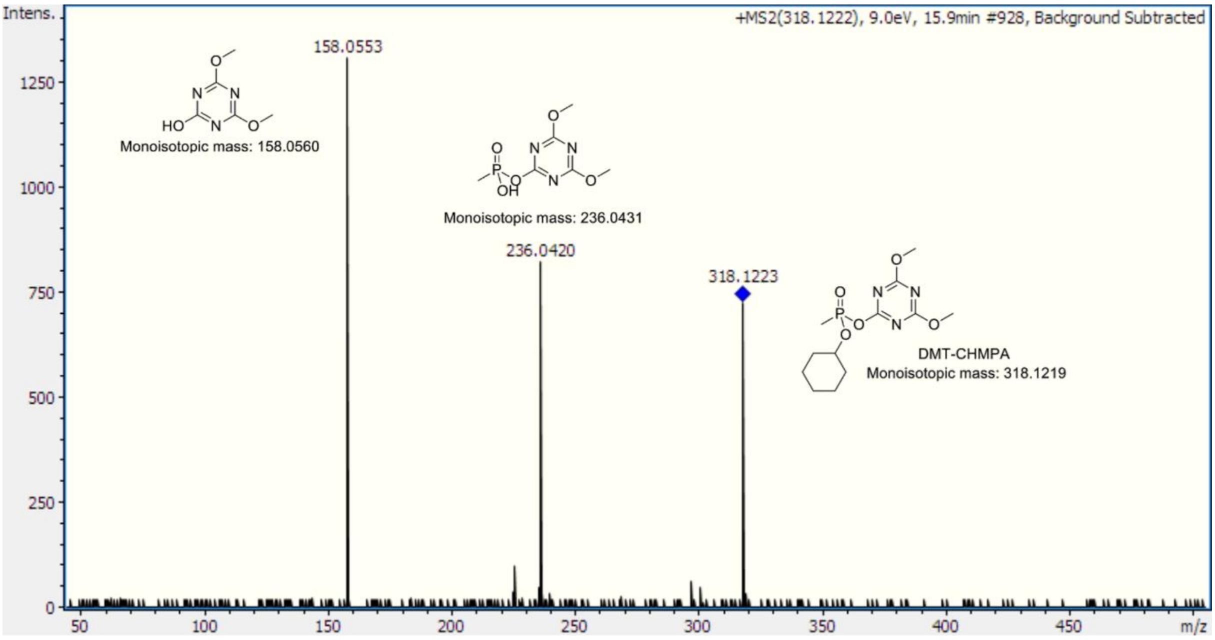


Figure 5. Product ion spectrum of DMT-CHMPA.

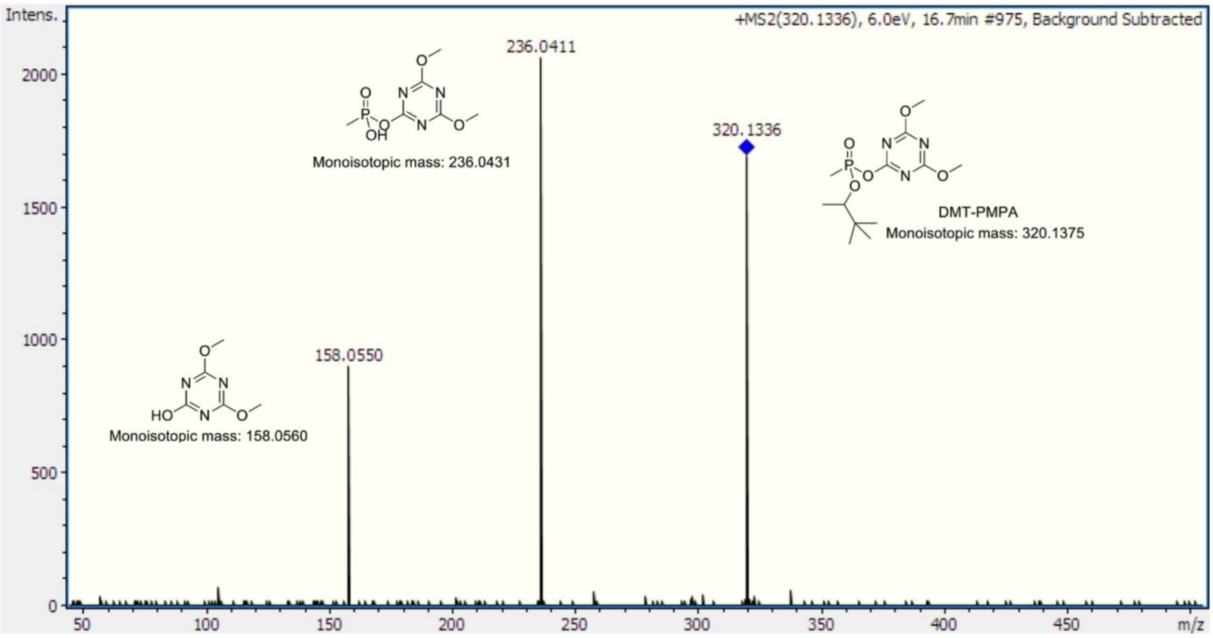


Figure S6. Product ion spectrum of DMT-PMPA.

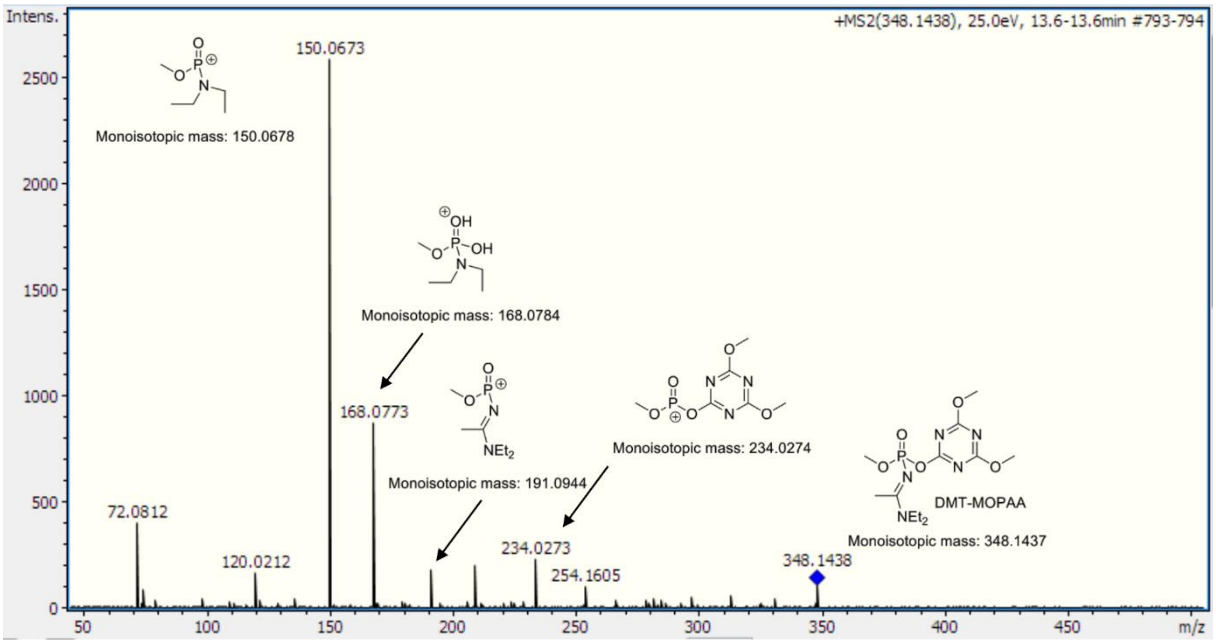


Figure S7. Product ion spectrum of DMT-MOPAA.

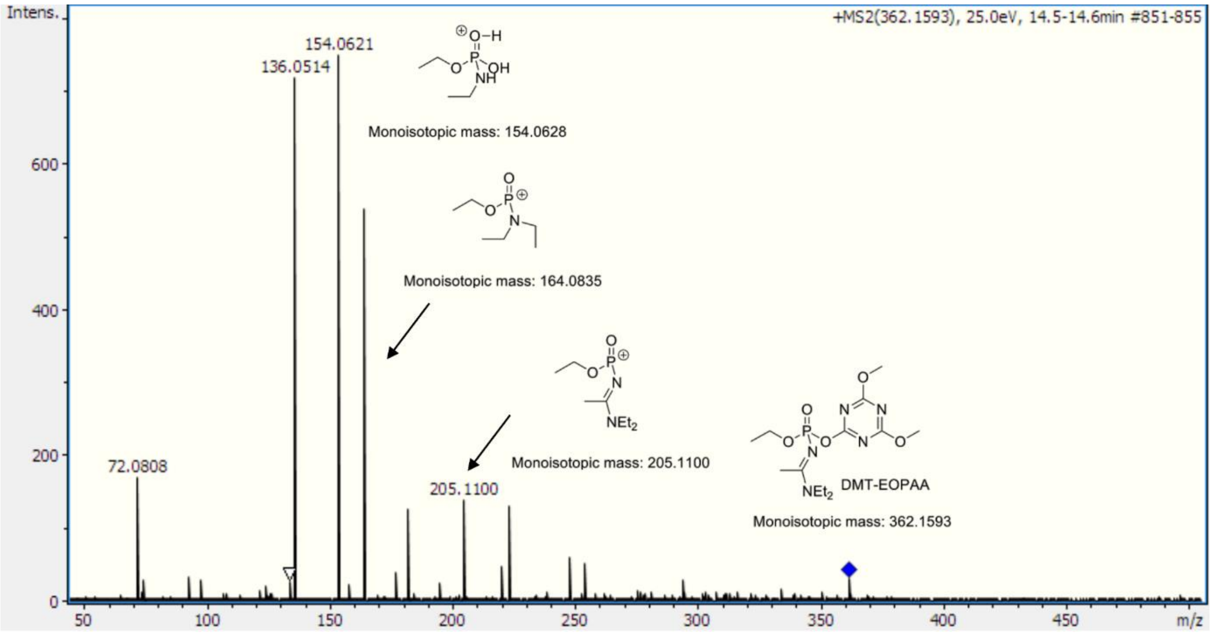


Figure S8. Product ion spectrum of DMT-EOPAA.

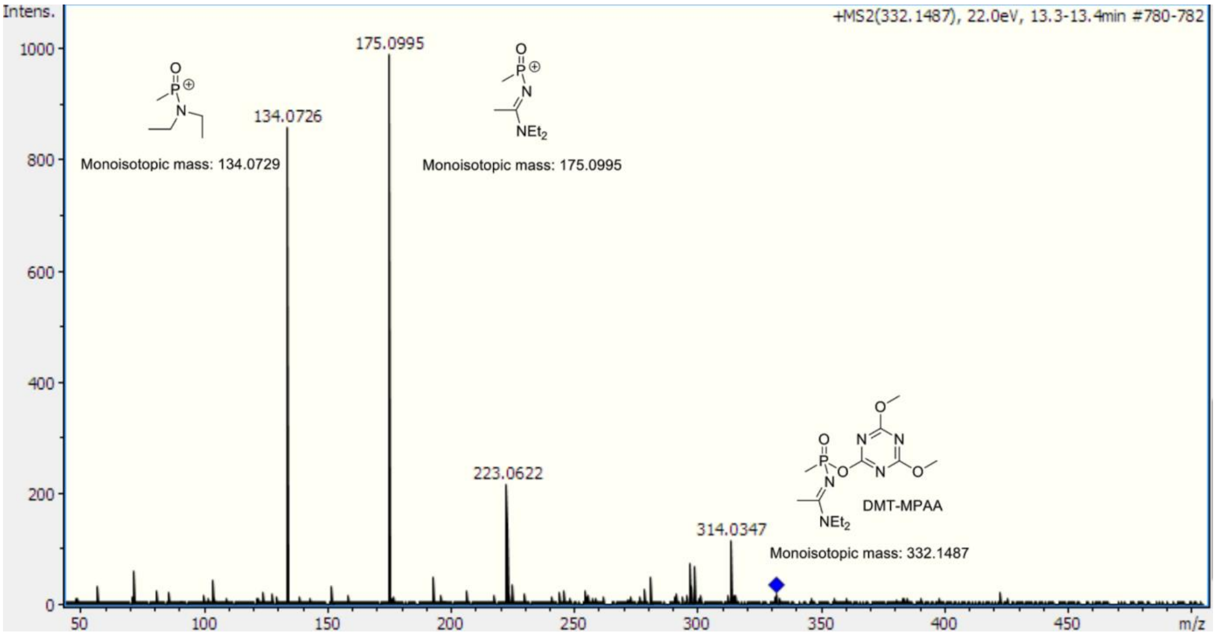


Figure S9. Product ion spectrum of DMT-MPAA.

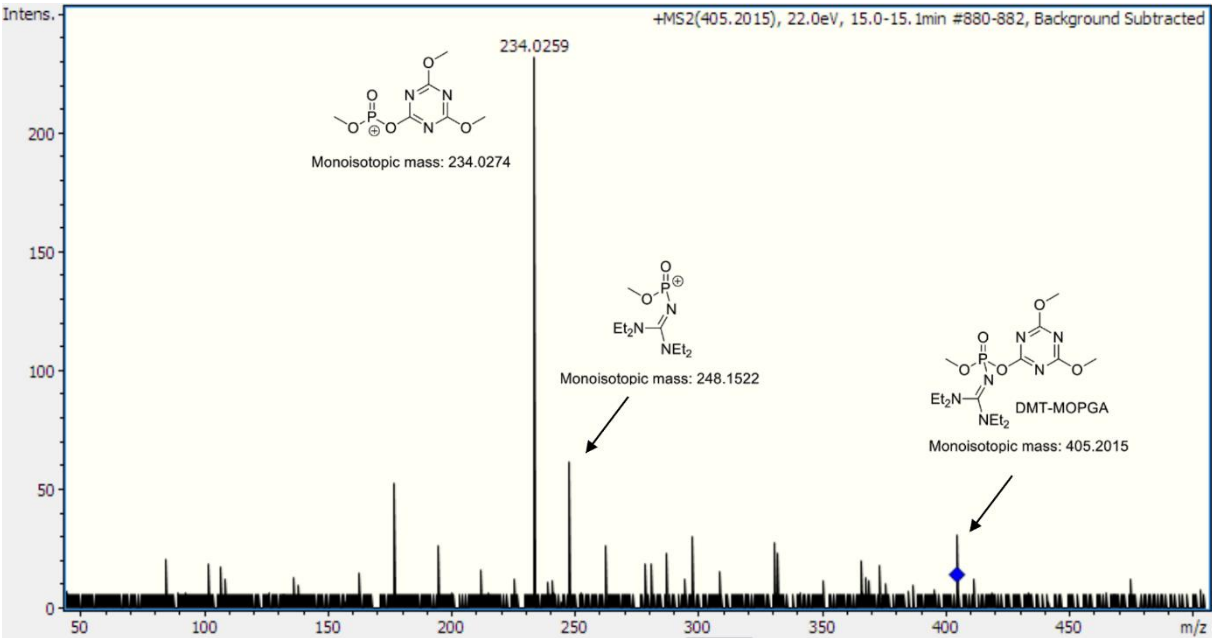


Figure S10. Product ion spectrum of DMT-MOPGA.

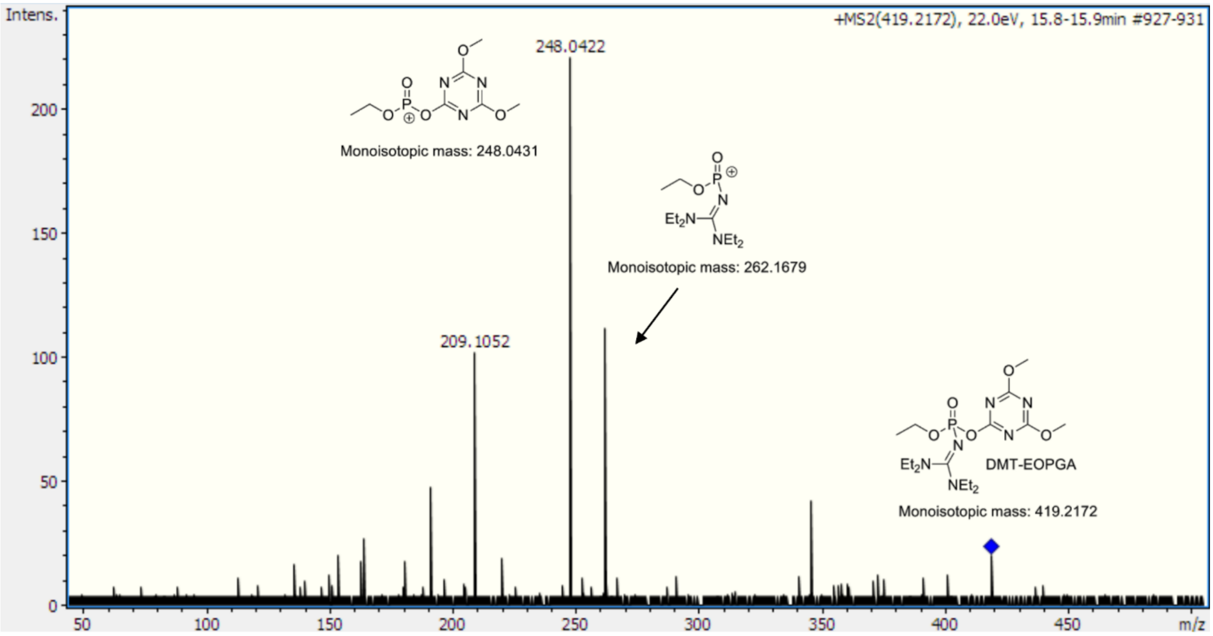
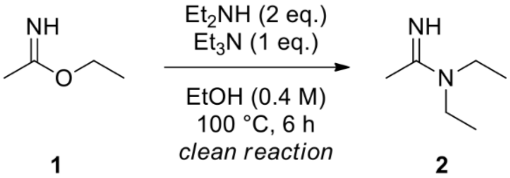


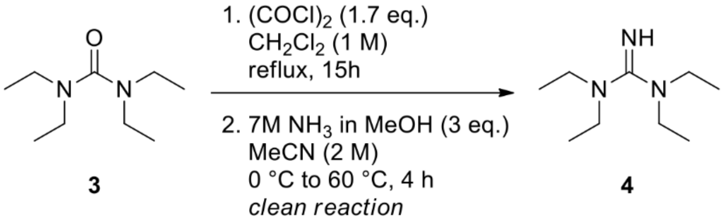
Figure S11. Product ion spectrum of DMT-EOPGA.

Chapter 2: Synthetic method

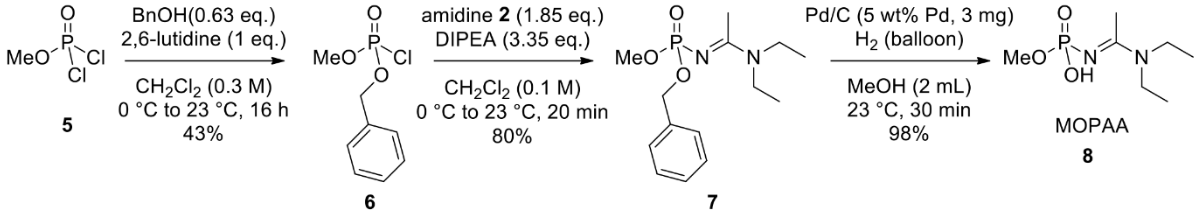
General: All reactions sensitive to air or moisture were carried out under nitrogen atmosphere in dry solvents under anhydrous conditions, unless otherwise noted. Dry CH_2Cl_2, DMF, and toluene were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). All other reagents were used as supplied. Analytical thin-layer chromatography (TLC) was performed using E. Merck Silica gel 60 F 25 pre-coated plates, 0.25 mm. Preparative thin-layer chromatography was performed using Merck silica gel 60 F 254 pre-coated plates, 0.5 mm. Flash chromatography was performed using 40-50 μm Silica Gel 60N (Kanto Chemical Co., Inc.), unless otherwise noted. ^1H and ^{13}C NMR spectra were recorded on JEOL JNM-ECX-600 spectrometer. Chemical shifts were reported in ppm on the δ scale relative to $CHCl_3$ ($\delta = 7.26$ for ^1H NMR), $CDCl_3$ ($\delta = 77.0$ for ^{13}C NMR), as internal references. Signal patterns are indicated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broaden peak. High resolution mass spectra were measured on Bruker Impact HD Q-TOF mass spectrometer (Bruker, Billerica, MA, USA) via an electrospray ionization mode. Ionization was performed in positive mode.



Amidine 2. Et_2NH (3.72 mL, 36.0 mmol) was added to a solution of ethyl acetimidate **1** (2.22 mL, 18.0 mmol) and Et_3N (2.50 mL, 18.0 mmol) in EtOH (45 mL) at room temperature. The reaction mixture was stirred at 100 °C for 6 h, and was quenched with 30% aqueous NaOH (20 mL) at room temperature. The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (20 mL x3). The combined organic layers were dried over Na_2SO_4, filtered and concentrated. The residue was slightly brownish but almost pure in ^1H NMR. Therefore, it was used for the reactions described below without further purification. ^1H NMR (600 MHz, $CDCl_3$) δ 1.12 (6H, t, NCH_2CH_3), 2.09 (3H, s, CH_3), 3.30 (4H, q, NCH_2CH_3).



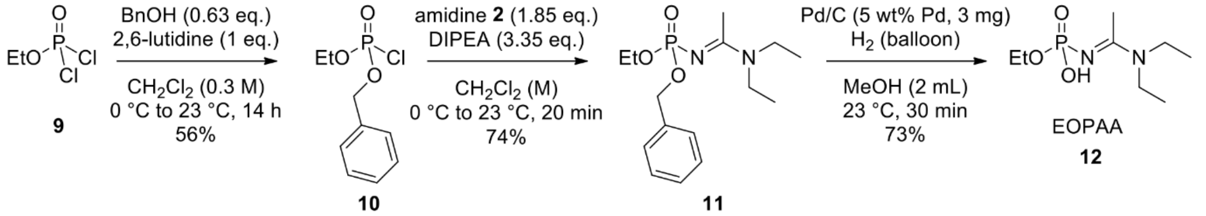
Guanidine 4. Oxalyl chloride (1.46 mL, 17.1 mmol) was slowly added to a solution of tetraethylurea **3** (1.9 mL, 10.0 mmol) in CH_2Cl_2 (10 mL) at 23 °C. The reaction mixture was stirred at 60 °C for 15 h, and was concentrated. The yellow oil residue was used for the next reaction without further purification. 7M NH_3 in MeOH (4.30 mL, 30.1 mmol) was added to a solution of the crude residue in MeCN (5.7 mL) at 0 °C. The reaction mixture was stirred at 60 °C for 4 h, and was concentrated. Et_2O (35 mL) and 13% aqueous NaOH (15 mL, saturated with K_2CO_3) were added at 0 °C. The organic layer was separated, and the aqueous layer was extracted with Et_2O (10 mL x3). The combined organic layers were dried over K_2CO_3, filtered and concentrated. The residue was slightly yellowish but almost pure in ^1H NMR. Therefore, it was used for the reactions described below without further purification. ^1H NMR (600 MHz, $CDCl_3$) δ 1.05 (12H, t, NCH_2CH_3), 3.09 (8H, q, NCH_2CH_3).



Benzyl methyl phosphorochloridate 6. 2,6-lutidine (356 μL, 3.00 mmol) and benzyl alcohol (194 μL, 1.89 mmol, R_f 0.45 for CH_2Cl_2/acetone 100:1) were slowly added to a solution of methyl phosphorodichloridate **5** (300 μL, 3.00 mmol) in CH_2Cl_2 (10 mL) at 0 °C. The reaction mixture was stirred at 23 °C for 15 h, and was concentrated. The residue was speedily purified by flash column chromatography on silica gel (12 g, charged with hexane/CH_2Cl_2 5:1, eluted with hexane/CH_2Cl_2 1:1) to afford benzyl methyl phosphorochloridate **6** (285 mg, 1.29 mmol) in 43% yield: pale yellow oil; ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.88 (3H, d, POC*H*$_3$), 5.21 (2H, dt, OC*H*$_2$C$_6$H$_5$), 7.39 (5H, aromatic).

Benzyl methyl (1-(diethylamino)ethylidene)phosphoramidate 7. Amidine **2** (44 μL, 0.325 mmol) and ^{i}Pr$_2$NEt (102 μL, 0.589 mmol) were slowly added to a solution of benzyl methyl phosphorochloridate **6** (39 mg, 0.176 mmol, R_f 0.5 for CH_2Cl_2/acetone 100:1) in CH_2Cl_2 (1.8 mL) at 0 °C. The reaction mixture was stirred at 23 °C for 15 h, and was concentrated. The residue was purified by flash column chromatography on silica gel (3 g, CH_2Cl_2/acetone 3:1) to afford benzyl methyl (1-(diethylamino)ethylidene)phosphoramidate **7** (42 mg, 0.141 mmol) in 80% yield: colorless oil; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.11 (3H, t, NCH$_2$C*H*$_3$), 1.17 (3H, t, NCH$_2$C*H*$_3$), 2.36 (3H, s, CC*H*$_3$), 3.32 (2H, q, NC*H*$_2$CH$_3$), 3.47 (2H, q, NC*H*$_2$CH$_3$), 3.65 (3H, d, POC*H*$_3$), 5.02 (2H, m, OC*H*$_2$C$_6$H$_5$), 7.31–7.40 (5H, aromatic); ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.3, 13.9, 19.7, 42.8, 43.6, 52.9, 67.6, 127.7, 128.4, 137.6, 165.7; HRMS (ESI) calcd for $C_{14}H_{24}N_2O_3P$ [M+H]$^+$ 299.1519, found 299.1520.

***O*-methyl (1-(diethylamino)ethylidene)phosphoramidic acid (MOPAA) 8**. A solution of benzyl methyl (1-(diethylamino)ethylidene)phosphoramidate **7** (9.6 mg, 0.032 mmol, R_f 0.3 for CH_2Cl_2/acetone 3:1) and Pd/C (5 wt% Pd on carbon, 3 mg) in MeOH (2 mL) was exposed to H_2 atmosphere (1 atm, balloon), and was stirred for 30 min at room temperature. Then, the reaction mixture was filtered through a disposable syringe filter, DISMIC JP (0.20 μm, 0.9 cm^2, ADVANTEC TOYO KAISHA, Ltd., Tokyo, Japan) with MeOH (5 mL). The filtrate was concentrated to afford *O*-methyl (1-(diethylamino)ethylidene)phosphoramidic acid (MOPAA) **8** (6.6 mg, 0.031 mmol) in 98 % yield: colorless oil; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.11 (3H, t, NCH$_2$C*H*$_3$), 1.27 (6H, m, NCH$_2$C*H*$_3$), 2.60 (3H, s, CC*H*$_3$), 3.44 (2H, q, NC*H*$_2$CH$_3$), 3.58 (3H, d, POC*H*$_3$), 3.80 (2H, q, NC*H*$_2$CH$_3$); ^{13}C NMR (150 MHz, CDCl 3) δ 12.2, 13.3, 17.2, 45.0, 46.3, 52.9, 165.3; HRMS (ESI) calcd for $C_7H_{18}N_2O_3P$ [M+H]$^+$ 209.1050, found 209.1048.

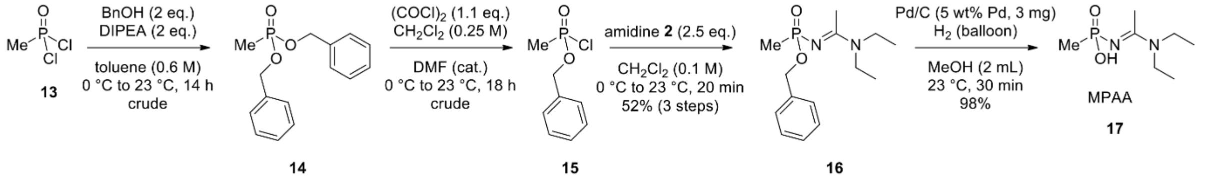


Benzyl ethyl phosphorochloridate 10. 2,6-lutidine (186 μL, 1.60 mmol) and benzyl alcohol (104 μL, 1.00 mmol, R_f 0.45 for CH_2Cl_2/acetone 100:1) was slowly added to a solution of ethyl phosphorodichloridate **9** (188 mg, 1.60 mmol) in CH_2Cl_2 (3.3mL) at 0 °C. The reaction mixture was stirred at 23 °C for 15 h, and was concentrated. The residue was purified by flash column chromatography on silica gel (8 g, charged with hexane/CH_2Cl_2 5:1, eluted with hexane/CH_2Cl_2 2:1) to afford benzyl ethyl phosphorochloridate **10** (132 mg, 0.560 mmol) in 56% yield: colorless oil; ^{1}H

NMR (600 MHz, $CDCl_3$) δ 1.38 (3H, dt, $POCH_2CH_3$), 4.27 (2H, m, $POCH_2CH_3$), 5.20 (2H, dt, $OCH_2C_6H_5$), 7.39 (5H, aromatic).

Benzyl ethyl (1-(diethylamino)ethylidene)phosphoramidate 11. Amidine **2** (26.6 μL, 0.198 mmol) and $^{i}Pr_2NEt$ (62 μL, 0.358 mmol) was slowly added to a solution of benzyl ethyl phosphorochloridate **10** (25 mg, 0.107 mmol, R_f 0.7 for CH_2Cl_2/acetone 100:1) in CH_2Cl_2 (1.07 mL) at 0 °C. The reaction mixture was stirred at 23 °C for 15 h, and was concentrated. The residue was purified by flash column chromatography on silica gel (2 g, CH_2Cl_2/acetone 6:1 to 3:1) to afford benzyl ethyl (1-(diethylamino)ethylidene)phosphoramidate **11** (25 mg, 0.079 mmol) in 74% yield: colorless oil; ^1H NMR (600 MHz, $CDCl_3$) δ 1.11 (3H, t, NCH_2CH_3), 1.16 (3H, t, NCH_2CH_3), 1.27 (3H, t, $POCH_2CH_3$), 2.36 (3H, s, CCH_3), 3.31 (2H, q, NCH_2CH_3), 3.47 (2H, q, NCH_2CH_3), 4.02 (2H, m, $POCH_2CH_3$), 5.02 (2H, m, $OCH_2C_6H_5$), 7.28–7.40 (5H, aromatic); ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.3, 14.0, 16.4, 19.7, 42.8, 43.6, 62.0, 67.5, 127.7, 128.4, 137.8, 165.7; HRMS (ESI) calcd for $C_{15}H_{26}N_2O_3P$ $[M+H]^+$ 313.1676, found 313.1679.

***O*-ethyl (1-(diethylamino)ethylidene)phosphoramidic acid (EOPAA) 12**. A solution of benzyl ethyl (1-(diethylamino)ethylidene)phosphoramidate **11** (10 mg, 0.032 mmol) and Pd/C (5 wt% Pd on carbon, 3 mg) in MeOH (2 mL) was exposed to H_2 atmosphere (1 atm, balloon), and was stirred for 30 min at room temperature. Then, the reaction mixture was filtered through a disposable syringe filter, DISMIC JP (0.20 μm, 0.9 cm^2, ADVANTEC TOYO KAISHA, Ltd.) with MeOH (5 mL). The filtrate was concentrated to afford *O*-ethyl (1-(diethylamino)ethylidene)phosphoramidic acid (EOPAA) **12** (5.2 mg, 0.023 mmol) in 73 % yield: colorless oil; ^1H NMR (600 MHz, $CDCl_3$) δ 1.11 (3H, t, NCH_2CH_3), 1.23 (3H, t, $POCH_2CH_3$), 1.28 (6H, m, NCH_2CH_3), 2.64 (3H, s, CCH_3), 3.45 (2H, q, NCH_2CH_3), 3.83 (2H, q, NCH_2CH_3), 3.96 (2H, dq, $POCH_2CH_3$); ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.2, 13.3, 16.6, 17.2, 45.1, 46.5, 61.7, 165.4; HRMS (ESI) calcd for $C_8H_{20}N_2O_3P$ $[M+H]^+$ 223.1206, found 223.1205.

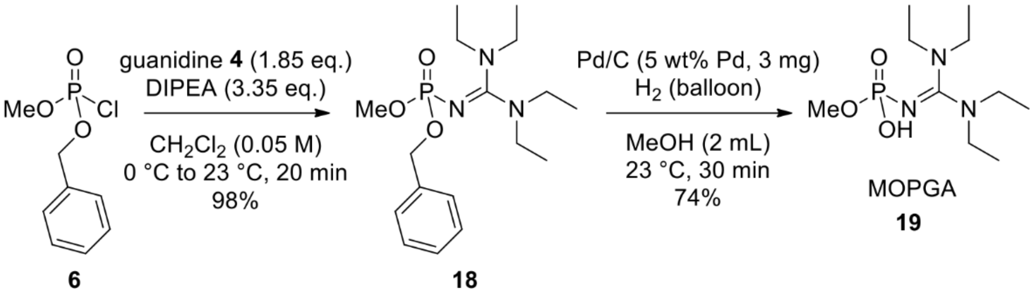


Dibenzyl methylphosphonate 14. $^{i}Pr_2NEt$ (379 μL, 2.17 mmol) and benzyl alcohol (225 μL, 2.17 mmol) was slowly added to a solution of methylphosphonic dichloride **13** (100 μL, 1.09 mmol) in toluene (1.8 mL) at 0 °C. The reaction mixture was stirred at 23 °C for 18 h, and was filtered with celite and hexane, followed by concentration. Hexane/Et_2O (1:1, 3 mL) was added and washed 0.03 M aqueous NaOH (3 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The residue was used for next reaction without further purification: pale yellow oil; ^1H NMR (600 MHz, $CDCl_3$) δ 1.48 (3H, d, $POCH_3$), 5.01 (4H, dt, $OCH_2C_6H_5$), 7.35 (10H, aromatic).

Benzyl methylphosphonochloridate 15. Oxalyl chloride (103 μL, 1.20 mmol) and dry DMF (1.0 μL, catalytic) was slowly added to a solution of crude dibenzyl methylphosphonate **14** (R_f 0.5 for $CHCl_3$/MeOH 100:1) in CH_2Cl_2 (4.4 mL) at 0 °C. The reaction mixture was stirred at 23 °C for 18 h, and was concentrated. The residue was used for next reaction without further purification: pale yellow oil; ^1H NMR (600 MHz, $CDCl_3$) (impure) δ 2.00 (3H, d, $POCH_3$), 5.20 (2H, dt, $OCH_2C_6H_5$), 7.39 (5H, aromatic)

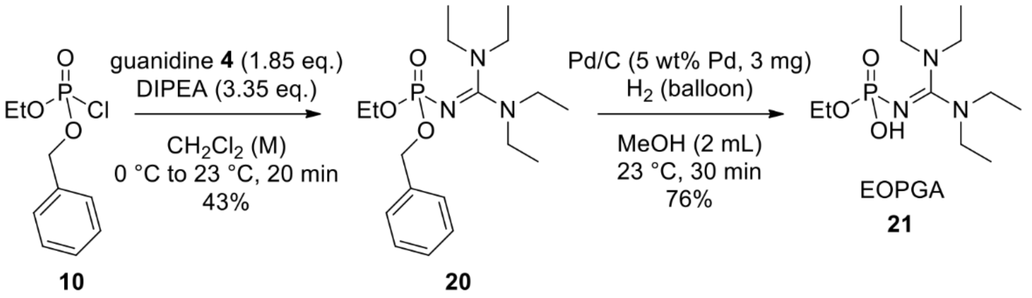
Benzyl *N*-(1-(diethylamino)ethylidene)methylphosphonamidate 16. Amidine **2** (311 μL, 2.73 mmol) was slowly added to a solution of crude benzyl methylphosphonochloridate **15** (R_f 0.3 for CH_2Cl_2/acetone 1:1) in CH_2Cl_2 (10 mL) at 0 °C. The reaction mixture was stirred at 23 °C for 30 min, and was concentrated. The residue was purified by flash column chromatography on silica gel (10 g, CH_2Cl_2/acetone 3:1) to afford benzyl *N*-(1-(diethylamino)ethylidene)methylphosphonamidate **16** (160 mg, 0.567 mmol) in 52% yield (3 steps): colorless oil; ^1H NMR (600 MHz, $CDCl_3$) δ 1.09 (3H, t, NCH_2CH_3), 1.16 (3H, t, NCH_2CH_3), 1.47 (3H, d, PCH_3), 2.38 (3H, s, CCH_3), 3.30 (2H, q, NCH_2CH_3), 3.44 (2H, q, NCH_2CH$_3$), 5.00 (2H, m, OCH_2C$_6$H$_5$), 7.25–7.39 (5H, aromatic); ^{13}C NMR (150 MHz, CDCl$_3$) δ 12.4, 14.0, 14.8, 15.7, 19.7, 42.6, 43.3, 65.9, 127.5, 127.7, 128.4, 138.1, 164.5; HRMS (ESI) calcd for $C_{14}H_{24}N_2O_2P$ [M+H]$^+$ 283.1570, found 283.1568.

***O*-ethyl (1-(diethylamino)ethylidene)phosphoramidic acid (MPAA) 17**. A solution of benzyl *N*-(1-(diethylamino)ethylidene) **16** (10 mg, 0.035 mmol) and Pd/C (5 wt% Pd on carbon, 3 mg) in MeOH (2 mL) was exposed to H$_2$ atmosphere (1 atm, balloon), and was stirred for 30 min at room temperature. Then, the reaction mixture was filtered through a disposable syringe filter, DISMIC JP (0.20 μm, 0.9 cm^2, ADVANTEC TOYO KAISHA, Ltd.) with MeOH (5 mL). The filtrate was concentrated to afford *O*-ethyl (1-(diethylamino)ethylidene)phosphoramidic acid (MPAA) **17** 6.7 mg, 0.035 mmol) in 98 % yield: colorless oil; ^{1}H NMR (600 MHz, CDCl$_3$) δ 1.26 (6H, t, NCH$_2$CH_3), 1.52 (3H, d, PCH_3), 2.69 (3H, s, CCH_3), 3.45 (2H, q, NCH_2CH$_3$), 3.78 (2H, q, NCH_2CH$_3$); ^{13}C NMR (150 MHz, CDCl$_3$) δ 12.0, 13.3, 16.7, 17.2, 18.1, 44.7, 46.3, 164.8; HRMS (ESI) calcd for $C_7H_{18}N_2O_3P$ [M+H]$^+$ 193.1100, found 193.1097.



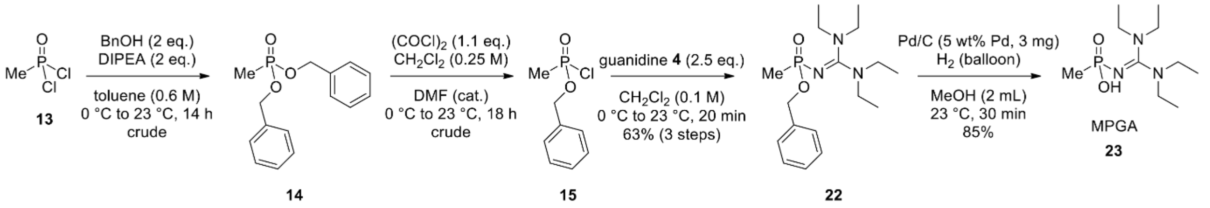
Benzyl methyl (bis(diethylamino)methylene)phosphoramidate 18. Guanidine **4** (34 μL, 0.198 mmol) and ^{i}Pr$_2$NEt (62 μL, 0.358 mmol) was slowly added to a solution of benzyl methyl phosphorochloridate **6** (25 mg, 0.107 mmol, *R*$_f$ 0.5 for CH$_2$Cl$_2$/acetone 100:1) in CH$_2$Cl$_2$ (1.1 mL) at 0 °C. The reaction mixture was stirred at 23 °C for 15 h, and was concentrated. The residue was purified by flash column chromatography on silica gel (2 g, CH$_2$Cl$_2$/acetone 6:1 to 3:1) to afford benzyl methyl (bis(diethylamino)methylene)phosphoramidate **18** (27 mg, 0.103 mmol) in 97% yield: colorless oil; ^{1}H NMR (600 MHz, CDCl$_3$) δ 1.10 (12H, t, NCH$_2$CH_3), 3.29 (8H, q, NCH_2CH$_3$), 3.62 (3H, d, POCH_3), 5.02 (2H, m, OCH_2C$_6$H$_5$), 7.25–7.40 (5H, aromatic); ^{13}C NMR (150 MHz, CDCl$_3$) δ 13.1, 43.4, 52.8, 67.4, 127.7, 128.3, 137.9, 162.6; HRMS (ESI) calcd for $C_{17}H_{31}N_3O_3P$ [M+H]$^+$ 356.2098, found 356.2100.

***O*-methyl (bis(diethylamino)methylidene)phosphoramidic acid (MOPGA) 19**. A solution of benzyl methyl (bis(diethylamino)methylene)phosphoramidate **18** (8.1 mg, 0.023 mmol) and Pd/C (5 wt% Pd on carbon, 3 mg) in MeOH (2 mL) was exposed to H$_2$ atmosphere (1 atm, balloon), and was stirred for 30 min at room temperature. Then, the reaction mixture was filtered through a disposable syringe filter, DISMIC JP (0.20 μm, 0.9 cm^2, ADVANTEC TOYO KAISHA, Ltd.) with MeOH (5 mL). The filtrate was concentrated to afford *O*-methyl (bis(diethylamino)methylidene)phosphoramidic acid (MOPGA) **19** (4.5 mg, 0.017 mmol) in 74 % yield: colorless oil; ^{1}H NMR (600 MHz, CDCl$_3$) δ 1.22 (12H, t, NCH$_2$CH_3), 3.46(8H, q, NCH_2CH$_3$), 3.64 (3H, d, POCH_3); ^{13}C NMR (150 MHz, CDCl$_3$) δ 12.9, 29.8, 44.3, 52.6, 161.5; HRMS (ESI) calcd for $C_{10}H_{25}N_3O_3P$ [M+H]$^+$ 266.1628, found 266.1628.



Benzyl ethyl (bis(diethylamino)methylene)phosphoramidate 20. Guanidine **4** (19 μL, 0.099 mmol) and ^{i}Pr$_2$NEt (61 μL, 0.179 mmol) was slowly added to a solution of benzyl ethyl phosphorochloridate **10** (12 mg, 0.053 mmol, R_f 0.7 for CH_2Cl_2/acetone 100:1) in CH_2Cl_2 (540 μL) at 0 °C. The reaction mixture was stirred at 23 °C for 1 h, and was concentrated. The residue was purified by preparative thin-layer chromatography (width 20 cm, height 10 cm, CH_2Cl_2/acetone 2:1) to afford benzyl ethyl (bis(diethylamino)methylene)phosphoramidate **20** (8.5 mg, 0.023 mmol) in 43% yield: colorless oil; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.10 (12H, t, NCH$_2$C*H*$_3$), 1.24 (3H, t, POCH$_2$C*H*$_3$), 3.29 (8H, m, NC*H*$_2$CH$_3$), 4.01 (3H, m, POC*H*$_2$H$_3$), 5.02 (2H, d, OC*H*$_2$C$_6$H$_5$), 7.25–7.40 (5H, aromatic); ^{13}C NMR (150 MHz, $CDCl_3$) δ 13.1, 16.4, 43.4, 61.6, 67.3, 127.6, 128.3, 137.9, 162.5; HRMS (ESI) calcd for $C_{18}H_{33}N_3O_3P$ [M+H]$^+$ 370.2254, found 370.2257.

***O*-ethyl (bis(diethylamino)methylidene)phosphoramidic acid (EOPGA) 21**. A solution of benzyl ethyl (bis(diethylamino)methylene)phosphoramidate **20** (5.6 mg, 0.015 mmol) and Pd/C (5 wt% Pd on carbon, 3 mg) in MeOH (2 mL) was exposed to H_2 atmosphere (1 atm, balloon), and was stirred for 30 min at room temperature. Then, the reaction mixture was filtered through a disposable syringe filter, DISMIC JP (0.20 μm, 0.9 cm^2, ADVANTEC TOYO KAISHA, Ltd.) with MeOH (5 mL). The filtrate was concentrated to afford *O*-ethyl (1-(diethylamino)ethylidene)phosphoramidic acid (EOPAA) **12** (3.2 mg, 0.012 mmol) in 76 % yield: colorless oil; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.21 (12H, t, NCH$_2$C*H*$_3$), 1.23 (3H, t, POCH$_2$C*H*$_3$), 3.45 (8H, q, NC*H*$_2$CH$_3$), 4.01 (3H, m, POC*H*$_2$H$_3$), 8.56 (1H, br, PO*H*); ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.9, 16.7, 44.4, 61.3, 161.5; HRMS (ESI) calcd for $C_{11}H_{27}N_3O_3P$ [M+H]$^+$ 280.1785, found 287.1781.



Benzyl *N*-(bis(diethylamino)methylene)methylphosphonamidate 22. Crude benzyl methylphosphonochloridate **15** was synthesized same as above using methylphosphonic dichloride **13** (25 μL, 0.253 mmol). Guanidine **4** (122 μL, 0.632 mmol) was slowly added to a solution of crude benzyl methylphosphonochloridate **15** (R_f 0.3 for CH_2Cl_2/acetone 1:1) in CH_2Cl_2 (2.5 mL) at 0 °C. The reaction mixture was stirred at 23 °C for 30 min, and was concentrated. The residue was purified by flash column chromatography on silica gel (3 g, CH_2Cl_2/acetone 3:1 to 1:1) to afford benzyl *N*-(bis(diethylamino)methylene)methylphosphonamidate **22** (58 mg, 0.159 mmol) in 63% yield (3 steps): pale yellow oil; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.09 (12H, t, NCH$_2$C*H*$_3$), 1.47 (3H, d, PC*H*$_3$), 3.28 (8H, m, NC*H*$_2$CH$_3$), 5.01 (2H, m, OC*H*$_2$C$_6$H$_5$), 7.24–7.38 (5H, aromatic); ^{13}C NMR (150 MHz, $CDCl_3$) δ 13.2, 14.7, 15.7, 43.5, 43.6, 66.0, 127.6, 128.4, 138.3, 161.5; HRMS (ESI) calcd for $C_{17}H_{31}N_3O_2P$ [M+H]$^+$ 340.2148, found 340.2149.

***N*-(bis(diethylamino)methylene)methylphosphonamidic acid (MPGA) 23**. A solution of benzyl *N*-(bis(diethylamino)methylene)methylphosphonamidate **22** (8.7 mg, 0.026 mmol) and Pd/C (5 wt% Pd on carbon, 3 mg) in MeOH (2 mL) was exposed to H_2 atmosphere (1 atm, balloon), and was stirred for 30 min at room temperature. Then, the reaction mixture was filtered through a disposable syringe filter, DISMIC JP (0.20 μm, 0.9 cm^2, ADVANTEC TOYO KAISHA, Ltd) with MeOH (5 mL). The filtrate was concentrated to afford *N*-(bis(diethylamino)methylene)methylphosphonamidic acid (MPGA) **23** (5.4 mg, 0.022 mmol) in 85 % yield: colorless oil; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.22 (12H, t, NCH_2CH_3), 1.51 (3H, d, PCH_3), 3.45 (8H, m, NCH_2CH_3), 8.96 (1H, br, PO*H*); ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.9, 17.0, 17.8, 44.3, 161.3; HRMS (ESI) calcd for $C_{10}H_{25}N_3O_2P$ $[M+H]^+$ 250.1679, found 250.1677.