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REVIEW ARTICLE

A review on analysis methods for nerve agent hydrolysis products

Intan Soraya Che Sulaiman[1] · Buong Woei Chieng[1] · Fellyzra Elvya Pojol[2] · Keat Khim Ong[1,3] · Jahwarhar Izuan Abdul Rashid[3] · Wan Md Zin Wan Yunus[4] · Noor Azilah Mohd Kasim[1,3] · Norhana Abdul Halim[3] · Siti Aminah Mohd Noor[3] · Victor Feizal Knight[1]

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Abstract

Purpose This review article provides an overview of the background of nerve agents (NAs) and their hydrolysis products. The analytical techniques that are used for the analysis of hydrolysis products are also discussed. The analysis methods include the chromatographic, mass spectrometric, electrophoretic, and sensing technologies that are currently available for the detection of exposure to a number of hydrolysis products of NAs.

Methods All reviewed information was gathered through a detailed search of Scopus, PubMed, and the World Wide Web using relevant keywords, e.g., NA, hydrolysis products, and methylphosphonic acid.

Results Most analyses of NA hydrolysis products still rely on chromatographic techniques such as gas and liquid chromatography. Analyses based on mass spectrometry are now fairly routine and can be further improved by providing analyses with precise and accurate masses and faster scan speeds that provide further gains in sensitivity. Although these techniques have high selectivity and sensitivity, most of them are rarely applied to real samples or suffer from destructive real sample analysis. In addition, there is still a lack of studies on the development of portable sensors for the detection of NA hydrolysis products.

Conclusions Although the analysis of NA hydrolysis products has experienced a transition from qualitative analysis toward rapid and quantitative identification, portable, and fast sensing technologies have not been well established or investigated extensively. In the future, more analysis methods are expected to be developed and may involve the development of sensors and lab-on-chip devices that should address specificity, sensitivity, stability, response time, and repeatability/reproducibility.

Keywords Nerve agent · Hydrolysis · Detection · Methylphosphonic acid · Alkyl methylphosphonic acid

Introduction

A nerve agent (NA) is an extremely toxic chemical that disrupts the transmission of nerve impulses via inhibition of acetylcholinesterase (AChE) activity through the phosphorylation process. This process causes acetylcholine (ACh) accumulation in the synaptic junctions, thereby hindering muscle relaxation. For that reason, the AChE enzyme is needed for relaxation of a muscle or organ after stimulation. Accumulation of ACh may cause overstimulation of central and peripheral cholinergic receptors to result in salivation, convulsions, paralysis, loss of consciousness, sweating, miosis, and death [1, 2]. Based on the structures of alkylphosphonic acid esters, NAs can be classified into two main groups: G- and V-series [3]. However, both types share a similar frame structure consisting of a phosphorus center that binds with one leaving group and one or two lipophilic groups. Additionally, a double bond exists between

✉ Keat Khim Ong
ongkhim@upnm.edu.my

1 Centre for Chemical Defence, Universiti Pertahanan Nasional Malaysia (National Defence University of Malaysia), Sungai Besi Camp, 57000 Kuala Lumpur, Malaysia

2 Department of Defence Science, Faculty of Defence Science and Technology, National Defence University of Malaysia, Sungai Besi Camp, 57000 Kuala Lumpur, Malaysia

3 Department of Chemistry and Biology, Centre for Defence Foundation Studies, National Defence University of Malaysia, Sungai Besi Camp, 57000 Kuala Lumpur, Malaysia

4 Centre for Tropicalisation, National Defence University of Malaysia, Sungai Besi Camp, 57000 Kuala Lumpur, Malaysia

phosphorus and oxygen [4]. Due to toxicity effects, hydrolysis is used to destroy or neutralize these NAs. This process involves degradation of the parent compounds, resulting in the formation of various hydrolysis products with significantly reduced toxicity. Degradation products may also be used as an indicator of the use of NAs in the environment and may play a key role in assessing the degree of parent agent contamination [3].

Current technologies used in NA research have received special attention for the development of detection methods for their degradation products [5–7], medical treatment of NA poisoning [8, 9], and improvement of in-field and portable detection systems [10, 11]. The G-series is the first and oldest family of NAs. These compounds were first synthesized by German scientists in the 1930s and 1940s. Later, the V-series, a second family of NAs that uses the code letter "V" for "venom", was invented in the United Kingdom in the 1950s [2]. Additionally, a new isomer of VX agent called Russian VX (RVX) or VR was developed by the Union of Soviet Socialist Republics (USSR) [12]. In comparison to the G-series, V-series NAs possess low volatility and, therefore, are considered persistent NAs. For example, VX is approximately 2000 times less volatile than GB (sarin) [2]. Generally, all NAs are viscous liquids, but V-series agents tend to be persistent on surfaces. Due to their nonvolatile properties, V-series agents persist in the environment where they are dispersed and present a dangerous threat to ecosystems via the transdermal exposure route [2, 13]. Moreover, their persistence in the environment may be subject to abiotic and biotic degradation mechanisms [14].

Hence, continuous research and development in the analysis of NA hydrolysis products is crucial to identify the persistence of breakdown products and to monitor their degree of contamination in the environment to assist in clean-up processes and for public safety [14, 15]. This review provides a comprehensive summary of up-to-date information on recent developments in the analysis of different NA hydrolysis products.

V-series nerve agents

Historically, the first V-series NAs were synthesized in the 1950s and used as pesticides and chemical warfare agents [16]. Basically, V-series NAs contain sulfur and are classified as alkylphosphonothiolates [14]. V-series NAs include VX, VR, VG, VM, and VE (Fig. 1). However, two main V-series agents that are often discussed in the literature are VX and VR.

VX vapor is reported to be more toxic than the G-series NAs, with an LCt_{50} of 10 mg/min/m^3 compared to LCt_{50} of 400 mg/min/m^3 for tabun (GA), the most toxic NA in the G-series. The vapor density value of VX is 9.2, which is higher than those of other G-series members, and its stability and persistence in soil and water to accumulate in low grounds have been proven [15]. VX possesses acute toxicity even at very low doses, and its splashed liquid may persist for months in cold environments. Most V-series NAs are colorless and odorless liquids. However, VR and VM have been reported to range from colorless to yellow liquids, and VR also smells like fried sunflower seeds [16]. V-series NA liquids are lipophilic (soluble in lipids or oils), and their routes of exposure are through skin absorption [17]. Although both VX and VR have the same molecular formula ($C_{11}H_{26}NO_2PS$), differences in their structures have a significant impact on their physical and chemical properties. Compared to VX, VR was reported to be more persistent in

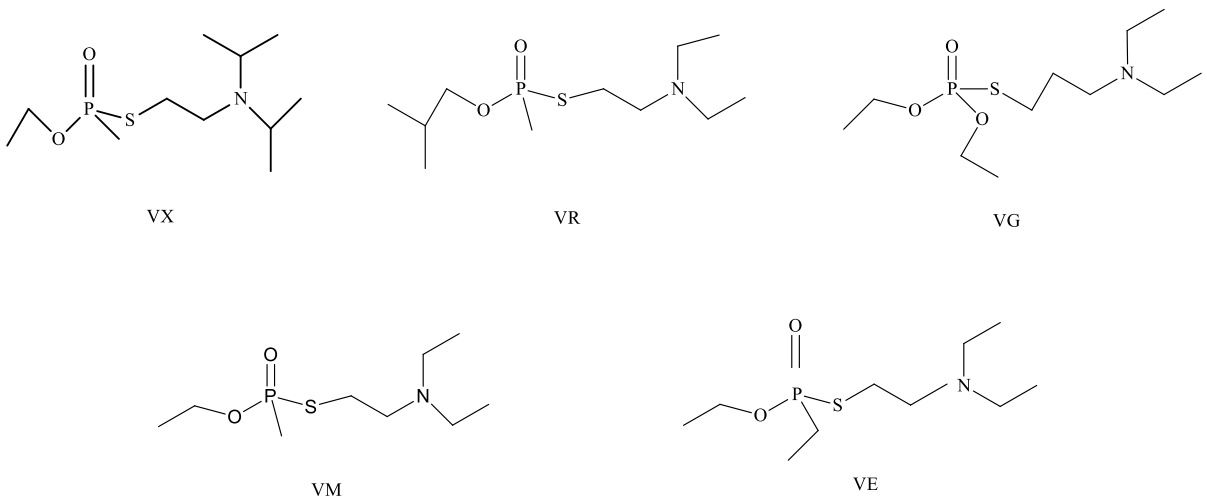

Fig. 1 Well-known members of the V-series of nerve agents

the environment, with a half-life of 12.4 days compared to 4.78 days for VX [18].

As mentioned earlier, V-series NAs are hazardous when organisms are exposed through skin contact. VX is the most potent NA, and even small drops of this poison may cause death. According to Rembovskiy et al. [19], the toxicity of VX when compared to sarin (GB) is approximately 170 times higher via skin exposure, 10 times higher via oral administration, and 2 times higher via inhalation. V-series compounds have unique molecular structures with varied transformations due to their polyfunctionality and high reactivity properties. This helps the compounds to be simultaneously incorporated and reacted with multicomponent matrices, whereas the involved reaction comprises multiple active centers in the same molecule. The most persistent hazardous hydrolysis products of VX are bis[2-(diisopropylamino) ethyl] disulfide (1) and *S*-[2-(diisopropylamino) ethyl] methylphosphonothioate (2) [19]. Compound 1 was the chemical compound detected on the face of murder victim Kim Jong-Nam and on the hands of the murderer [20]. In this incident, Kim Jong-Nam was killed by two suspected women when they rubbed something on his face separately within 7 s. When Kim rubbed his face, he felt pain in his eye before suddenly dying 20 min later. Police suggested that once Kim rubbed his face, VX entered his system through his eyes and caused death. A laboratory report suggested that Kim was killed with VX, as the agent was found in his body together with six other toxic chemicals, namely 2-(diisopropylamino) ethyl chloride, 2-(diisopropylamino) ethanethiol, *O*-ethyl methyl- phosphonothioic acid, ethyl methylphosphonic acid, bis(2-diisopropylaminoethyl) sulfide, bis(2-diisopropylaminoethyl) disulfide, and 2-(dimethylamino) ethanol [20]. In this murder case, the murderers may have used a modified binary system of VX, which was developed for safety purposes during storage [20]. Respective precursors involve in the reaction are relatively nontoxic in their single form, but if there are combined, they become active VX [20]. According to the authorities, two precursors may be used to form VX. Both precursors are identified as ethyl methylphosphonic acid from murderer's t-shirt and VX, 2-(diisopropylamino) ethyl chloride, 2-(diisopropylamino)ethanethiol, and bis(2-diisopropylaminoethyl) disulfide from another murderer's t-shirt and finger nails. This strongly suggests that both precursors may be employed as a binary system to form VX [20].

Nevertheless, MPA is less toxic than its parent agent, but its toxicity remains a great concern. In vivo toxicity studies of MPA in mice have demonstrated that MPA possesses low acute oral toxicity but is mildly irritating to rabbits' and humans' skin and eyes [21].

Dalton et al. [22] reported that the penetration rate of VX across the skin in the presence of water is greater than that across dry skin. The presence of water was shown to alter the diffusion rate of VX on wetted skin by changing its permeability coefficient [22]. The important characteristics of NA hydrolysis products, such as nonvolatile, polar, and water soluble, have provided a convenient platform for their direct detection and the indirect detection of their parent agents [23].

G-series nerve agents

The G-series is the first and oldest family of NAs. The first NA ever synthesized was GA (tabun) in 1936. GB (sarin) was developed next in 1938, followed by GD (soman) in 1944, and finally, the more obscure GF (cyclosarin) in 1948 [13]. Figure 2 shows the chemical structures of G-series NAs.

GB and GD have greater volatility and reactivity to water than VX and persist for a shorter time in the environment [24]. GB is an odorless and colorless volatile liquid at room temperature. It is the most volatile of the G-series agents [12]. Contrary to other toxic agents, protective clothing is ineffective when coming in contact with GB. It is assumed that clothes reduce the evaporation of GB, thereby increasing its effective dose. Another compound in the G-series is GD, which is also a colorless liquid in its pure form [12, 25]. Its volatility, intermediate between that of GA and GB, is high enough to make its vapors hazardous. It is less water-soluble and more lipid-soluble than the other G-series agents, resulting in more rapid dermal penetration and greater toxicity [26].

Hydrolysis products of nerve agents

Organophosphate NAs are extremely toxic such that their primary application is as chemical warfare agents. NAs may undergo degradation processes, including hydrolysis, photolysis, oxidation, and microbial degradation [14]. When these substances are dispersed in public places, it is usually difficult to identify them directly using on-site detection because of their ready decomposition by hydrolysis to alkyl methylphosphonic acids (AMPAs). AMPAs are comparatively safer to detect and can be further hydrolyzed to MPA. MPA, which forms slowly via hydrolysis of AMPAs, is a stable hydrolysis product. This acid is resistant to photolysis, hydrolysis and biodegradation because the bond form between carbon and phosphorus in the MPA molecule cannot be cleaved or metabolized by plants or animals [27].

Primary hydrolysis products of organophosphonate NAs include ethyl methylphosphonic acid (EMPA), the hydrolysis product of VX; isobutyl methylphosphonic acid (iBMPA), the hydrolysis product of VR; isopropyl methylphosphonic acid (iPMPA), the hydrolysis product of GB; pinacolyl

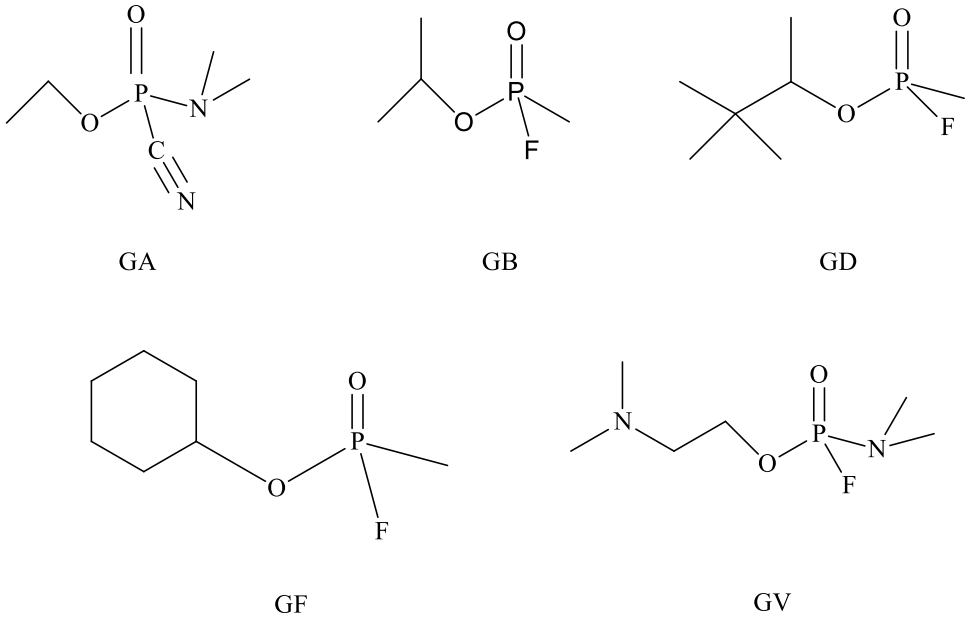

Fig. 2 The G-series of nerve agents

methylphosphonic acid (PMPA), the hydrolysis product of GD; cyclohexyl methylphosphonic acid (CMPA), the hydrolysis product of GF [28]; and *O*-ethyl *N*,*N*-dimethylamidophosphoric acid (EDMPA), the hydrolysis product of GA [14]. As used herein, "hydrolysis product" may refer to a metabolite produced by introduction of the NA to a living organism or may refer to a nonphysiological breakdown product.

Neutralization of NAs via base hydrolysis is one of the methods currently employed for NA destruction. During the hydrolysis process, sodium hydroxide is added as a base to facilitate neutralization of the acidic product of the reaction, thereby neutralizing its toxicity [13]. In addition, NA compounds may be subjected to decomposition reactions when reacting with water through the hydrolysis process. The hydrolysis products possess better stability and persistence in the environment than their parent compounds.

Hydrolysis of NAs through nucleophilic substitution at the phosphorus center appears to follow an associative pathway, for which two pathways exist (Fig. 3). The addition–elimination mechanism involves the presence of a pentacoordinate phosphorane intermediate resulting in the triple-well shape of the potential energy surface. It can then be described as a two-step process consisting of intermediate formation and its further decomposition. The direct-displacement pathway proceeds through a single S_N2-like transition state directly toward products, which is described by a double-well energy profile. The approach of the nucleophilic hydroxide ion is accompanied by expulsion of the leaving group. Independent of the number of

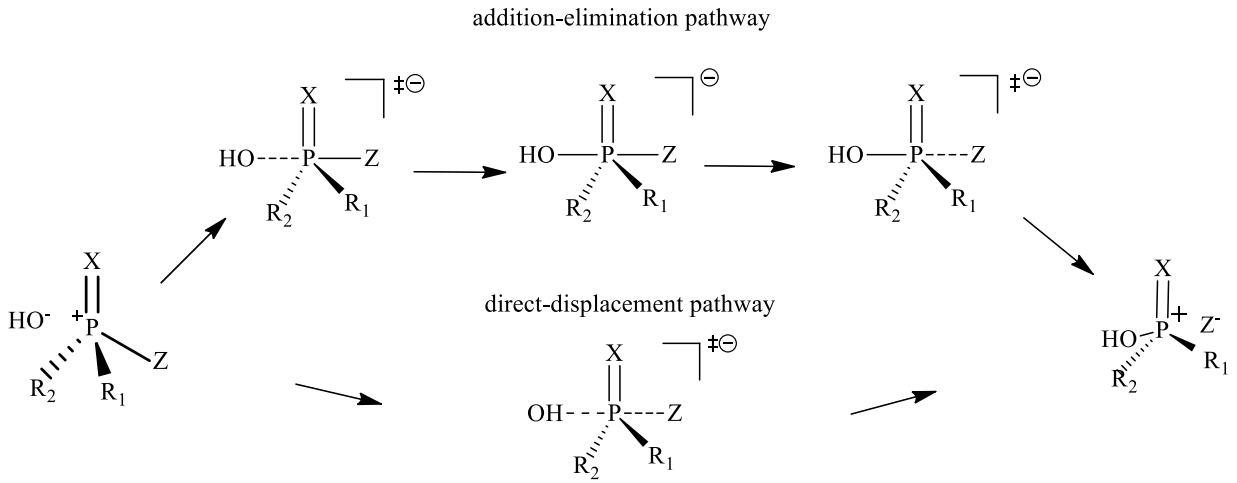

Fig. 3 Variants of S_N2-like nucleophilic substitution at the phosphorus center [28]

steps along the hydrolysis pathway, the favorable mechanism involves entering and leaving groups positioned on opposite sides of the plane formed by the three remaining atoms bonded to the phosphorus center [29].

Due to their labile properties, once NAs are exposed to the environment, they degrade rapidly with water via hydrolysis to yield corresponding alkyl phosphonic acids, phosphonothioic acids, and various alkyl amino ethanol compounds [23, 30]. Briefly, the hydrolysis reaction between phosphorus atoms and a leaving group of the agent compound forms AMPA, which then finally decomposes to MPA via hydrolysis of the alkoxy group [30].

The hydrolysis of V-series NAs is more complex. For example, VX contains hydrolytically labile P–S, P–O, and C–S bonds. Hydrolysis of VX could occur via three different pathways, P–S, P–O, and C–S bond cleavage, to produce various hydrolysis products (Fig. 4) [31]. The dominant bond cleavage pathway depends on the pH and concentration of the NA. For example, a 0.01 M solution of VX in water produces ethyl methylphosphonic acid (EMPA) via P–S cleavage and *S*-(2-diisopropylaminoethyl) methylphosphonothioate (EA 2192) via P–O cleavage in a ratio of ~ 6.5:1. Cleavage of the S–C bond form ethyl methylphosphonothioic acid (EMPTA) is usually a minor pathway. The pathway of most concern with respect to the environment is cleavage of the P–O bond because EA 2192 possesses high toxicity by systemic routes of administration [32].

In addition, temperature and pH also play important roles in influencing the rate of hydrolysis. A decrease in temperature has facilitated the long persistence of NAs in water [15]. Vucinic et al. [15] reported that the half-life of VX was varied in seawater in accordance with the temperature. At ambient temperature (25 °C), the half-life of VX is approximately 5–14 days; however, at 4 °C, the hydrolysis rate is slower; thus, it lasts longer in the environment, up to several years. According to the authors, the different behavior of VX is also related to pH conditions [15]. At an acidic pH value of 5, the half-life of VX is approximately 100 days, whereas at an alkaline pH of 8, the half-life is 9 days. In addition, the type of hydrolysis product formed is reported to be influenced by pH conditions. At a pH value of 5, the formed EMPA and MPA are more likely to persist in the environment; however, at pH values of 8, *S*-(2-diisopropylaminoethyl)methylphosphonic acid becomes more favorable [15]. Determination of the rate of hydrolysis may help in understanding the threat posed by NA use in related to their testing, production, and storage [18].

GB, GD and GF hydrolyze in the environment to their corresponding AMPAs (Fig. 5) [32]. V-series agents (VX, VR) are not typical substrates for phosphorylyl phosphatases, which probably explains the higher persistence of these agents

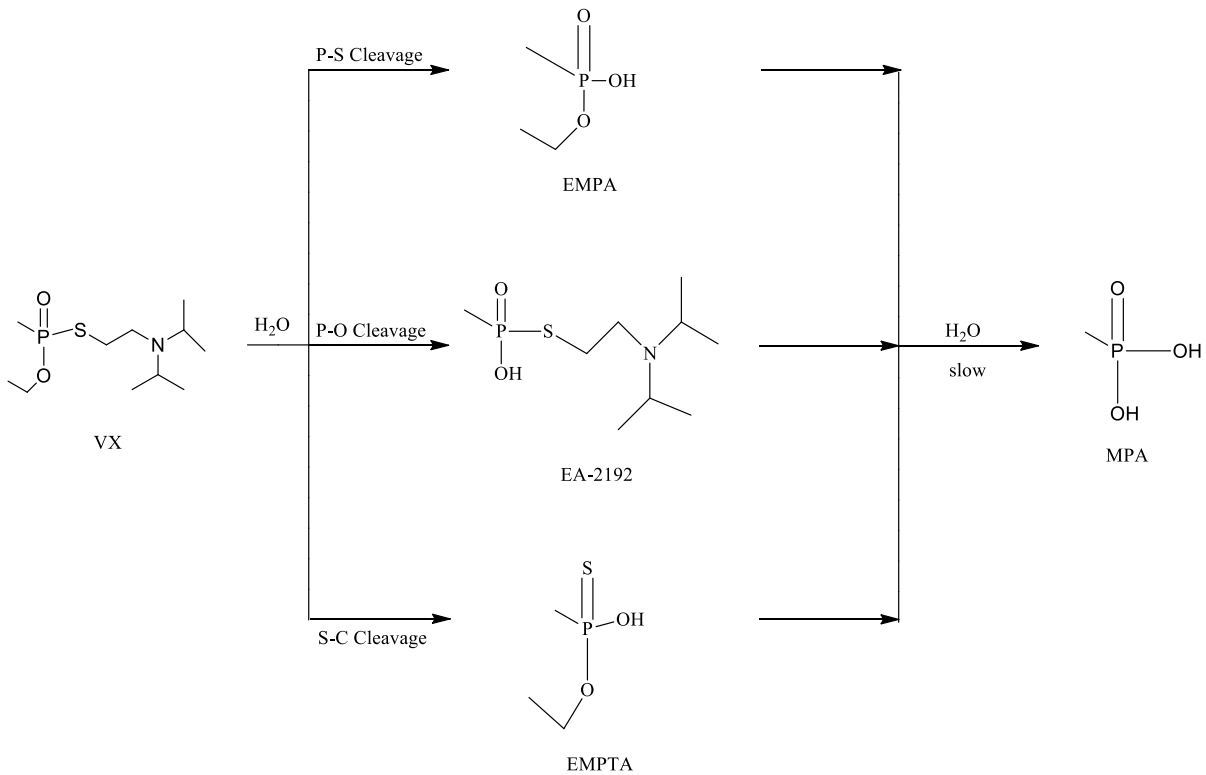

Fig. 4 Degradation pathways of the V-series agent VX [31]

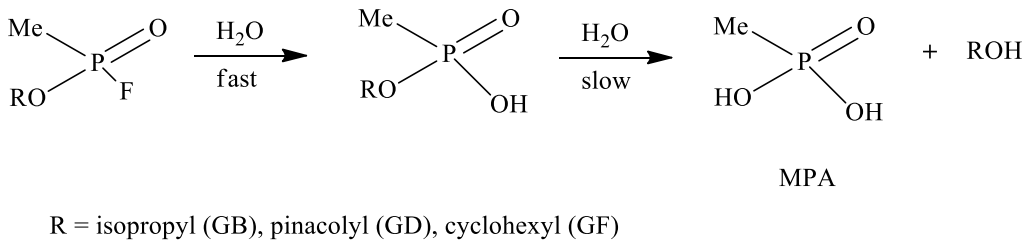

Fig. 5 Hydrolytic pathways for phosphonofluoridate nerve agents [32]

in organisms. The final hydrolysis product for both G- and V-series NAs is MPA, but its fraction is smaller than that of AMPAs [19].

GD (soman) hydrolyzes at a much slower rate in aqueous environments than GB but at a faster rate than GA. GD hydrolyzes under acidic and basic conditions to form the primary hydrolysis product PMPA, which slowly hydrolyzes to MPA. Hydrolysis of GD is slow under neutral conditions and may take up to 60 h at pH 6 and 25 °C. In diluted solutions, GD hydrolyzes within 1.8 min at pH 10.8 [33]. The hydrolysis of GA (tabun) is more complex. GA undergoes hydrolysis more rapidly under acidic and basic conditions than at a neutral pH. Depending on the pH of the environment, GA hydrolysis will proceed in one of two ways, as shown in Fig. 6 [34]. Under basic conditions (pH > 7), GA is hydrolyzed first to *O*-ethyl *N,N*-dimethylamidophosphoric acid (EDMPA) and cyanide anion finally to phosphoric acid. The same products are formed under neutral conditions, while ethylphosphoryl cyanidate and dimethylamine. are produced under acidic conditions (pH < 5).

Analysis methods of nerve agent hydrolysis products

Essentially, organophosphorus chemical warfare agents (OP-CWAs), including V-series NAs, have short lifespans in the human body and are hydrolyzed, metabolized or adducted to nucleophilic sites on macromolecules such as DNA and proteins [35]. Phosphonic acids are metabolites of OP-CWAs derived from hydrolysis and are present in the body after exposure to NA. Detection of these particular hydrolysis products may indicate exposure to their respective NAs. Gas chromatography (GC) and liquid chromatography (LC), combined with mass spectrometry (MS), are established techniques for the detection and identification of OP NAs and related hydrolysis compounds.

Chromatographic analysis coupled with mass spectrometry

Analysis of CWAs by chromatographic analysis coupled with MS has grown extensively over the past decade [36]. The coupling of MS to chromatographic techniques is desired due to its sensitivity and highly specific nature compared with other means of chromatographic detectors [37]. MS is designed to convert the analyte molecules to a charged (ionized) state, separate them according to their mass-to-charge ratio (m/z value) and finally be identified as the charged species [37]. Many types of MS have been developed that vary in the ionization sources and mass analyzers employed. In practice, the coupling of GC and LC to MS could extend the range of analytes detectable in a single sample. Separation, identification, and quantitation of NA hydrolysis products has been reported using GC coupled with MS (GC–MS) and GC coupled with tandem MS (GC–MS/MS). LC coupled with mass spectrometry (LC–MS) and LC coupled with tandem MS (LC–MS/MS) have also been used with either hydrophilic interaction LC (HILIC) or anion-exchange chromatography.

Gas chromatography–mass spectrometry

GC is historically the most widely used technique in the field, being applicable not only for volatile, non-polar, and easily vaporized compound. Analyses conducted by GC are usually coupled with specific detectors, such as electron capture detectors (ECDs), flame photometric detectors (FPDs), nitrogen phosphorus detectors (NPDs), and flame ionization detectors (FIDs). Different types of detectors can be used to detect different kinds of analytes with different advantages [38]. GC techniques are not as dependent on the differences in AMPA and MPA properties. AMPAs and MPA cannot be directly analyzed by GC because these compounds are considerably hydrophilic and nonvolatile and have very few chromophores in their structure, so their identification and quantitative analysis are usually carried out after derivatization to the respective less polar compounds [39].

Two of the most common methods of phosphonic acid derivatization for GC–MS analysis are silylation and methylation [40]. The most frequently used derivatizing agents are *N*-methyl-*N*-(tert-butyldimethylosilyl)trifluoroacetamide [26], *N*,*O*-bis(trimethylsilyl)acetamide [41] and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide [42], all of which are used to produce silyl derivatives, diazomethane,

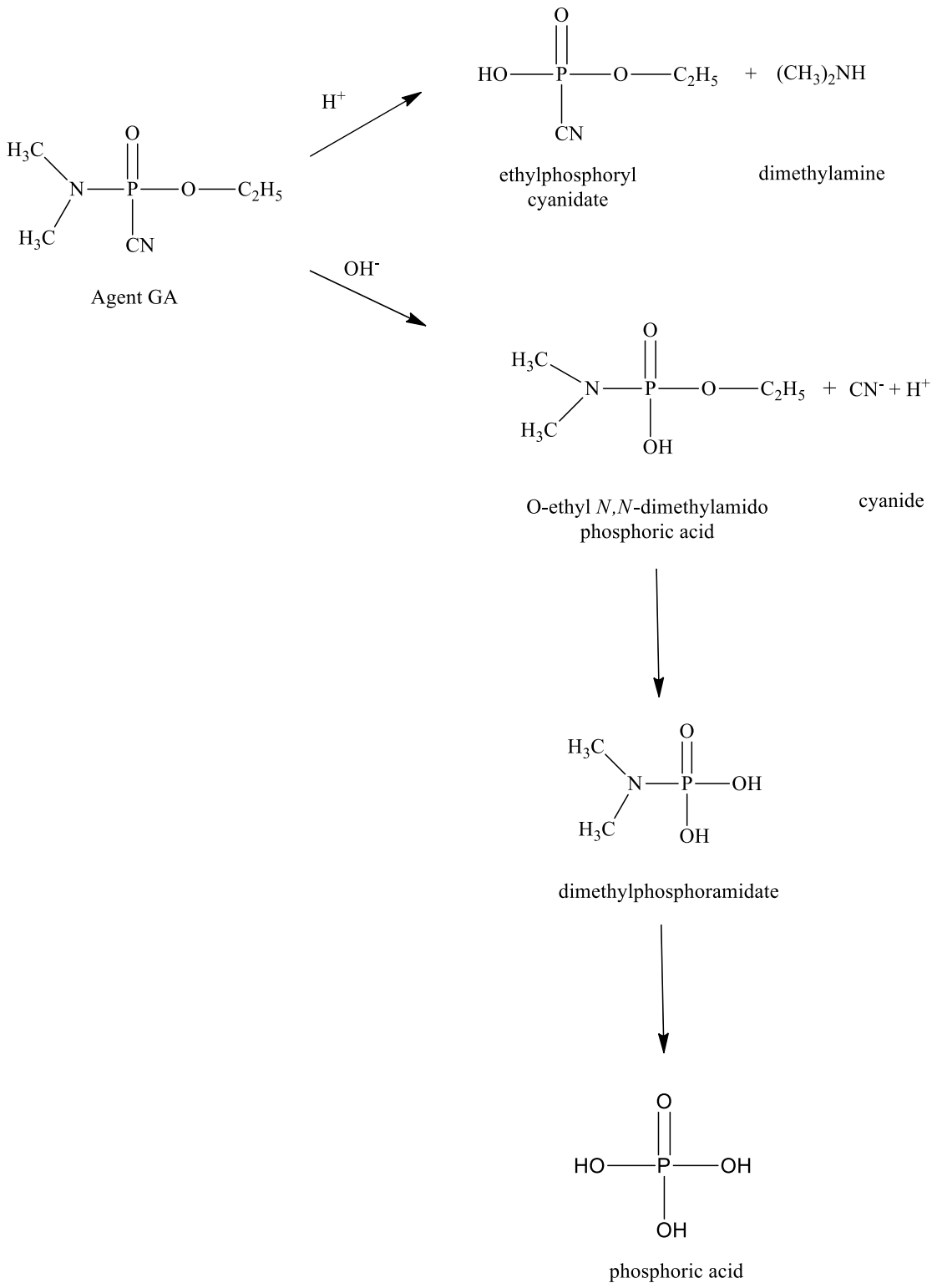

Fig. 6 Primary hydrolysis pathways of GA in the environment [34]

(trimethylosilyl)diazometane [43], trimethylphenylammonium hydroxide [44], and phenyltrimethylammonium bromide or iodide [45], which are used to produce methyl derivatives [46]. Diazomethane (CH_2N_2) has been the prime gold standard and powerful reagent of choice to carry out the derivatization of not only phosphonic acids but also species

such as carboxylic acids, aliphatic/aromatic alcohols, and even thiols. This is due to its reactivity under mild conditions and the fact that there are no other GC–MS-interfering byproducts generated during its use [47].

The derivatization step is a prerequisite that makes the analytical procedure tedious and prone to errors. Sometimes, a change of solvent is also required. This change results in increasing complexity and a time-consuming sample preparation procedure, which does not support the current trend of analytical chemistry aimed at decreasing time and increasing the simplicity of each analytical step, including sample preparation. In 2010, for the first time, nonderivatized AMPAs were analyzed using GC with FID. Połeć et al. [46] developed a direct GC-FID quantitative determination of nonderivatized AMPAs using a selective GC column, type CP-FFAP CB (Varian). The limit of quantification (LOQ), defined as the lowest concentration of the analyzed compound taken for analysis (on column), was estimated to be 6×10^4 ng/mL for EMPA, 3×10^4 ng/mL for iPMPA, 15×10^4 ng/mL for iBMPA, and 14×10^4 ng/mL for PMPA.

The most commonly used method for the analysis of derivatized alkyl monoesters of MPA is GC–MS with different ionization types. Ionization types include EI (electron ionization) [48], CI (chemical ionization) [49], and a TOF (time-of-flight) MS analyzer is also used [26, 50]. Subramaniam et al. [51] demonstrated the use of the direct derivatization of hydrolysis products using a new fluorinated phenyldiazomethane reagent [1-(diazomethyl)-3,5-bis(trifluoromethyl)benzene]. The sample preparation technique employed involves rapid direct derivatization (5 min) of acidified urine samples. The AMPAs were analyzed by GC–MS and MS/MS using negative ion chemical ionization. The selectivity and sensitivity of analyses performed in low- and high-resolution single ion monitoring MS mode were compared with those performed in multiple reactions monitoring MS/MS mode. The MS/MS technique offered the greatest sensitivity and selectivity of the tested MS techniques, with limits of detection (LODs) ranging from 0.5 to 1 ng/mL.

Kataoka and Seto [52] consolidated a series of methods for the quantitative determination of AMPAs and MPA in urine samples using strong anion-exchange (SAX) solid-phase extraction (SPE) followed by GC–MS after *tert*-butyldimethylsilyl (TBDMS) derivatization. A pretreatment method using SAX solid-phase extraction was developed to clean up urine samples, in which the sample was directly applied to a Bond Elut SAX cartridge, followed by elution of the AMPAs and MPA with 3% (v/v) methanolic ammonia, which were then derivatized and analyzed by GC–MS. The detection yields of the TBDMS derivatives of AMPAs and MPA were in the range of 61 to 97%.

An ideal derivatization method should involve the use of an inexpensive and readily available reagent, preferably one that is stable at ambient temperature and, most importantly, that poses minimal health and explosive hazards during its manipulation. Methylation of phosphonic acids (PMPA, CMPA and EMPA) employing trimethyloxonium tetrafluoroborate ($TMO{\cdot}BF_4$) for their qualitative detection and identification by GC–MS has been demonstrated by Valdez et al. [47] (Fig. 7). $TMO{\cdot}BF_4$ is a stable solid that can be stored at 4 °C and used immediately before every analysis, in contrast to the more commonly employed diazomethane-based methods that require fresh preparation of the reagent using potentially explosive synthetic routes. $TMO{\cdot}BF_4$ was found to conveniently settle to the bottom during the reactions and did not cause interference during the GC–MS analysis.

Recently, 2-(bromomethyl)naphthalene (BMN), a new derivatizing agent, was used for the determination of AMPAs such as EMPA, IMPA, and PMPA based on GC–TOF–MS using a UV femtosecond laser (267 nm) as the ionization source [50]. The LODs were < 1 ng/mL for these analytes. The use of BMN increased the volatility of the analytes for separation by GC and increased the ionization efficiency via the resonance-enhanced two-photon ionization (RE2PI) process as the result of the presence of a naphthalene functional group.

Liquid chromatography–mass spectroscopy

LC is applied as a separation and determination method for high-polarity and nonvolatile and/or thermally labile analytes. LC is often paired with classical detectors, such as UV detectors, fluorescence detectors and diode-array detectors (DADs). However, the use of an MS detection system is currently preferred because it presents high sensitivity and selectivity without derivatization [38]. The LC–MS/MS method has been extensively utilized in recent years for the quantification of NA hydrolysis products [29]. Due to its high sensitivity to hydrophilic compounds, analysis of NAs, which are relatively nonvolatile and highly polar, can be performed directly with minimal or no sample preparation. This technique employed coupling between LC and MS analysis, whereas a sample mixture was initially separated by LC before it was subjected to ionization and characterization using the *m/z* value and relative abundance using two mass spectrometers in series. LC–MS/MS has the ability to analyze complex mixtures with high molecular specificity and detection sensitivity to provide the structure identification of each component in the mixture. This tandem technique is an alternative to the GC–MS method, which has limitations in processing samples with polar and thermally labile properties.

A qualitative and quantitative screening technique for AMPAs in urine samples and extracts was reported by Lee and Lee [28] using LC–MS/MS in electrospray ionization mode (ESI). This tandem technique is an alternative to the

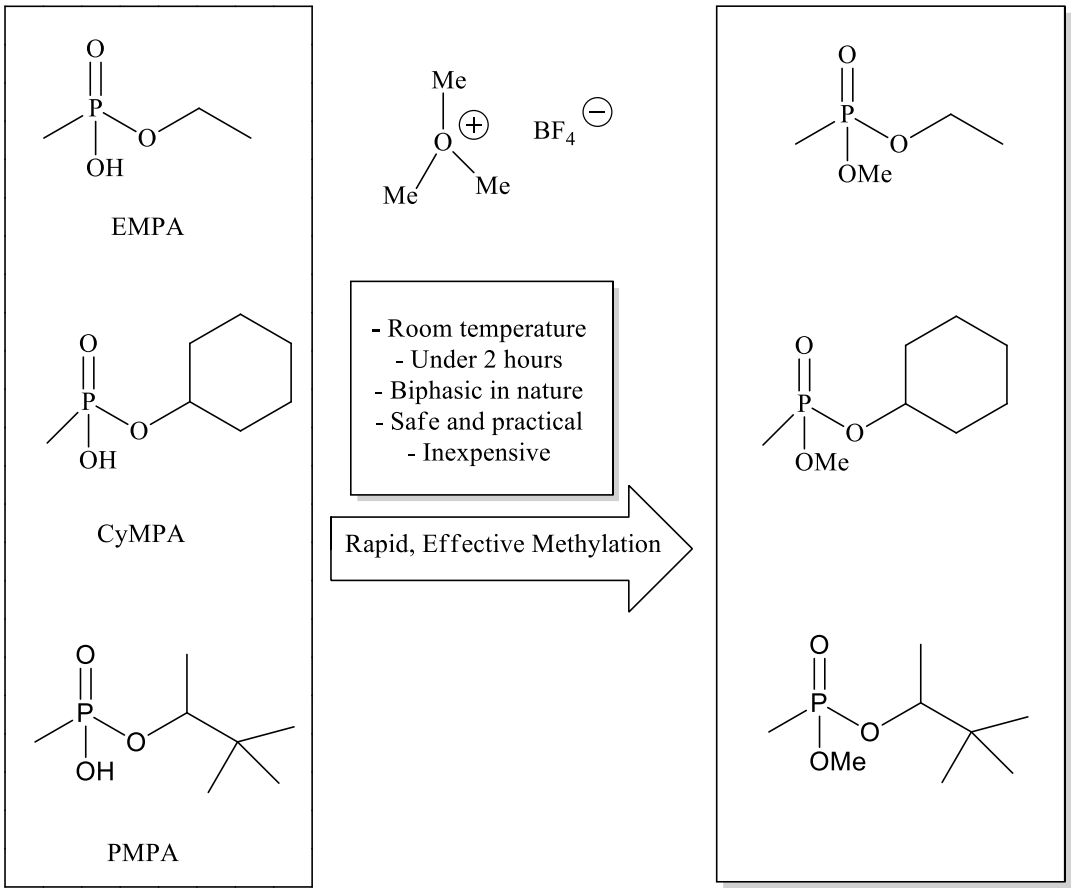

Fig. 7 Methylation of V- and G-series nerve agent hydrolysis products using TMO. BF4 protocol to yield methyl esters detectable by GC–MS [47]

GC–MS method, which has limitations in processing samples with polar and thermally labile properties [33]. By using precursor ion and selected reaction monitoring analysis, determination of the hydrolysis product AMPAs could be quantified with an LOD of 0.1 ng/mL. Hence, LC–ESI–MS/MS may be used to utilize a fragmentation ion of AMPA at *m*/*z* 95 to trace its common product ion in urine samples. In fact, urinary excretion of phosphonic acid metabolites from the body is the main elimination route resulting from NA exposure [28].

Additionally, blood samples can also be used to diagnose NA poisoning. Shaner et al. [53] determined the alkyl phosphonic acid metabolites in blood samples of human poisoning with VX and VR by HILIC coupled with MS/MS. In this study, the authors used dried blood spots (DBS) and microsampling devices as alternatives to conventional blood drawing to provide safe handling, prolong the stability, and provide user-friendly and economical techniques. Two hydrolysis products of the NAs EMPA (VX) and iBMPA (VR) were detected from DBS and microsampler devices. The LODs obtained when using DBS and the microsampler were 1.88 and 1.95 ng/mL for EMPA and 1.78 and 1.30 ng/mL for iBMPA, respectively. Furthermore, Yishai Aviram et al. [54] developed a new approach for the determination of phosphonic acids in blood using DBS on filter paper. Extraction was carried out after the blood had completely dried on the paper using methanol. The method is based on a simple sample preparation protocol, followed by use of LC–MS/MS targeted analysis [54]. The method has proven to be reliable for samples stored up to 35 days at room temperature, with a LOD for EMPA of 1 ng/mL. The LODs were 0.3 ng/mL for CMPA and 0.5 ng/mL for iPMPA.

As discussed earlier, the hydrophilicity of V-series NAs presents a great challenge for their isolation analysis due to the interaction of the metabolites with proteins in sample matrices. These hydrophilic analytes cannot be sufficiently retained on commonly used reversed-phase columns, and a high amount of energy is required for the collision-induced dissociation of these metabolites. To overcome this problem, Otsuka et al. [30] proposed the derivatization of hydrolysis products with pentafluorobenzylbromide (PFBBr) prior to LC–MS/MS analysis. Therefore, the addition of hydrophobic

groups to analytes may improve their retention on reversed-phase columns. This assay showed great accuracy, with LODs for EMPA and MPA of < 33 ng using this approach; in comparison, direct LC–MS/MS analysis without derivatization for similar metabolites gave an LOD of < 74 ng.

Baygildiev et al. [27] developed a time-efficient and simple technique for analyzing MPA in spiked natural waters using direct LC–MS/MS. From the experiment, the LOD for MPA in natural waters is 10 ng/mL. In addition, Owens and Koesters have reported quantitative analysis of MPA, EMPA, iPMPA, CMPA and PMPA in beverages by strata-X solid-phase extraction (SPE) cartridges using LC–MS/MS. Reasonable recoveries (> 50%) were achieved for the respective analytes, with an LOD of > 0.02 ng on-column [55]. In addition, Røen et al. [56] demonstrated an on-line SPE–LC–MS for determining various AMPAs (EMPA, iPMPA, iBMPA, CMPA and PMPA) at sub-ppb levels in aqueous soil extracts. The study employed zirconium dioxide (ZrO_2) as an SPE material due to its high recovery, minimal sample preparation and compatibility with LC–MS. ZrO_2, which consists of strong Lewis acid sites, has the ability to retain target analytes, even in media with high amounts of organic and inorganic interference. The method is compatible for screening primary NA hydrolysis products in aqueous soil extracts, with the LODs for AMPAs ranging from 0.05 to 0.5 ng/mL.

The use of high-resolution MS (HRMS) instruments has recently been introduced for quantitative analysis. One of the main attributes of HRMS analyzers is their accurate mass measurements, increasing the reliability of analyte detection by providing extra selectivity through the elemental composition of the parent and fragment ion spectra. The most common MS analyzers used in various analyte analyses are single quadrupole (Q), triple quadrupole (QqQ), ion trap (IT), hybrid quadrupole ion trap (QTrap), time of flight (TOF) and Orbitrap. Two SPE techniques for the analysis of glyphosate and methylphosphonic acid, which have structures similar to that of phosphonic acid-containing NAs, were developed by Wagner et al. [57]. Isotopically enriched analytes were used for quantitation by atmospheric pressure chemical ionization–quadrupole time-of-flight MS (APCI–Q–TOF–MS) that does not require derivatization. The developed methods are able to analyze a mixture of phosphonic acid-containing compounds in drinking water samples and meet the accuracy and precision required by the USEPA.

Previously, Papoušková et al. [58] analyzed *N*,*N*-dialkylaminoethane-2-thiols using µLC–MS via ESI-ion trap (IT) and characterization with a tandem quadrupole time of flight (QqTOF) mass spectrometer. The method employed ion-pairing chromatography using trifluoroacetic acid (TFA) as an ion-pairing agent. Due to the high polarity of analytes and the nature of *N*,*N*-dialkylaminoethane-2-thiols, which are highly liable to form disulfides, the separation process should be fast. Thus, the addition of TFA helps to increase the retention of analytes in the reverse-phase LC, thus allowing the separation of all isomers, with LODs of 100–500 ng/mL. In addition, Lee and Lee [30] developed a rapid analysis method for *N*,*N*-dialkylaminoethane-2-thiols using LC–MS/MS with selected reaction monitoring (SRM)-triggered QED-MS/MS. The method employed an analytical technique using the quantitation-enhanced data-dependent (QED) method. In a single analytical run, SRM analysis provided information-rich mass spectra that may simultaneously confirm the existence of compounds during the quantification process.

Later, in 2015, Lee and Lee [59] investigated the fragmentation of *N*,*N*-dialkylaminoethanesulfonic acids by LC–ESI–MS/MS using an IT mass spectrometer. V-series NAs degrade to form not only alkyl phosphonic acids but also phosphonothioic acids and various alkyl amino ethanol compounds [23]. *N*,*N*-Dialkylaminoethanesulfonic acid is a degradation product from the degradation of V-series agents in water. Theoretically, the hydrolysis of V-series agents may result in the formation of *N*,*N*-dialkylaminoethane-2-thiols and *O*-alkyl alkylphosphonic acids, precursors of their parent agent synthesis. Based on the detoxification pathway of V-series NAs, *N*,*N*-dialkylaminoethane-2-thiols may undergo oxidation to form more stable *N*,*N*-dialkylaminoethanesulfonic acids, which also act as markers for their parent agents and *N*,*N*-dialkylaminoethane-2-thiols [59]. According to the authors, 10 main fragmentation pathways of *N*,*N*-dialkylaminoethanesulfonic acids were observed. Most of these products were derived from the loss of alkyl groups attached to the nitrogen atoms.

Paper spray (PS)-MS has been used to analyze pesticides and herbicides in food and environmental samples, which are chemically similar to NAs [36]. PS-MS is an ambient ionization technique that requires little to no sample preparation, and analysis can be performed in seconds and can directly apply to complex biological and environmental samples. PS ionization coupled to HRMS (a quadrupole orbitrap) was used to identify and quantitate NA hydrolysis products in blood and urine [36]. Combining PS with MS capable of both high-resolution and tandem MS further improves selectivity. Five NA hydrolysis products, namely EMPA, iPMPA, iBMPA, CMPA and PMPA, were analyzed. PS-MS is capable of analyzing these hydrolysis products in both positive and negative ionization modes; however, higher sensitivity was found in negative ion mode. The LODs in negative ion mode ranged from 0.36 to 1.25 ng/mL for analysis of the hydrolysis products in both blood and urine.

Recently, an accumulation of NA markers in garden cress (*Lepidium sativum*) as a model plant object was investigated by Sarvin et al. [60], using an LC–QTOF hybrid system to determine MPA and alkyl methylphosphonates, which are specific markers of sarin, soman, VR and VX. Application

of the QTOF mass spectrometer with high mass resolution increased the accuracy of the measurements and achieved high reliability and sensitivity of MPA and AMPA determination in plant extracts. The LODs were found to be 1 ng/mL for IMPA, iBuMPA and PMPA; 10 ng/mL for EMPA; and 30 ng/mL for MPA.

The availability of HRMS instruments has increased LC–MS-based analysis in recent years, although in some cases, GC–MS methods are preferred when lower LODs are required [61].

Ion chromatography–mass spectrometry

There are several examples of IC tandemly coupled with suppressed conductivity detection for the determination of MPA in environmental water [62], and wastewater effluents [63] samples. Kingery and Allen [62] achieved an LOD of 0.1 ng/mL for MPA in environmental waters using IC with suppressed conductivity detection. However, their approaches were developed for the analysis of environmental waters that required sample preconcentration, which is not applicable in cases where the sample volume is limited (e.g., urine). The main problem associated with the IC method with suppressed conductivity when applied for the determination of MPA is that suppressed conductivity detection is not specific for the target analyte because all anionic species, such as inorganic and organic anions present in real samples, can influence separation [63]. This was very well demonstrated by Vermillion and Crenshaw [64], who reported the IC determination of MPA and iPMPA in soil extracts with suppressed conductivity detection. The conventional chemical-suppression IC successfully separated the analytes by ion-exchange mechanism by using quick and simple sample preparation process without special laboratory equipment [67]. Both MPA and iPMPA had 400 ng/g of soil of method detection limits in a 2-g soil sample [67].

A direct approach for the MPA determination in urine by IC–MS/MS was developed using a poly(styrene-*co*-divinylbenzene) (PS-DVB)-based anion exchanger by Baygildiev et al. [39]. The proposed approach does not require a large sample volume, complicated and laborious preconcentration or derivatization steps. The approach shows highly sensitive and reliable MPA determination with the lowest LOD (4 ng/mL) reported thus far for HPLC determination of MPA in urine.

Capillary electrophoresis

Over the past decade, considerable attention has been given to the application of capillary electrophoresis (CE) for the determination of hydrolysis products of NAs. CE is a simple, attractive alternative method to chromatographic techniques for the analysis of different phosphorus-containing compounds. A wide variety of detection methods, such as UV, fluorescence, laser-induced fluorescence, conductometric and amperometric methods, have been used in CE to determine different phosphorus compounds in biological and environmental matrices [65]. The increasing interest in CE stems from its high separation efficiency, short analysis time, low sample consumption and short preparation times, the last two factors being the technique's crucial benefits in the analysis of NAs. In terms of potential use in portable instruments, CE has one main advantage over the other separation methods. Separation by CE can be performed by the high voltage generated and does not require high-pressure pumps for operating as LC does. The generation of a high voltage in portable devices is more simple than the high pressure needed for chromatographic techniques [66]. Characterization and quantification of the hydrolysis products of NAs by CE, with an emphasis on the detection strategies, has been reviewed by several authors in the literature [67, 68]. CE analysis of small phosphorus-containing compounds, including organophosphates and hydrolysis products, was the subject of another review article [65].

Zi-Hui and Qin [69] reported for the first time that the NA hydrolysis products (PMPA, EMPA, and methane phosphonic acid) were analyzed directly using CE without a derivatization procedure. The extraction of hydrolysis products from human serum was performed by SPE using a molecularly imprinted polymer (MIP). SPE can be used to isolate and preconcentrate the analytes in complex samples. MIPs offer the possibility of achieving selective extraction and may represent an advance in conventional SPE materials. Following the SPE procedure, accurate analysis of the degradation products was carried out using CE directly. An LOD of 1×10^2 ng/mL and good linearity in the concentration range of 1×10^2–1×10^4 ng/mL were obtained [69].

Although conventional CE offers high sensitivity and selectivity, the method may not be reliably applied to on-site detection as for rapid screening owing to its size and technical complexity [42]. Hence, Ding et al. [70] developed an advanced technique by coupling microchip technology with CE for investigation of two AMPAs, namely EMPA and MPA. In this study, poly(dimethylsiloxane)-based microchips coupled with contactless conductivity detection were used to overcome the shortcomings of electrode fouling and unwanted side reactions. This recommended method offered shorter analysis times of less than 2 min, portability and low cost with minimal sample and reagent required.

The way in which the sample is injected plays an important role in making portable CE systems robust [67]. Most typically, the target analytes are introduced into the separation channel electrokinetically by applying a voltage between the sample reservoir and the grounded reservoir. Makarõtševa et al. [66] performed the analysis of NA hydrolysis products using a portable CE instrument with

various sample injection devices, such as a cross-sampler, a horizontal injection channel and a vertical injection channel. The cross-sampler required less manual operation, so it was more convenient to be used in field experiments. Unfortunately, the output of these injection devices may satisfy only the repeatability of migration times (1.7–6.0% RSD) but not provide sufficient precision for peak areas. Thus, only qualitative analysis was achieved in the study.

Sensors

Portable and rapid field detection of NA hydrolysis products is desirable for screening purposes and to augment standard laboratory methods. To improve on-site monitoring of NA hydrolysis products, a new detection method using sensors has been reported. Jenkins et al. [71] studied a polymer-based lanthanide luminescent sensor for detection of the hydrolysis product of the NA soman in water. The device was constructed using the luminescent lanthanide ion europium (Eu^{3+}) as the probe ion. The narrow excitation and emission peaks of lanthanide spectra (typically on the order of 0.01–1.00 nm full width at half maximum) provide highly sensitive and selective analyses. Detection of the NA is based on the changes that occur in the spectrum when the hydrolysis product PMPA is coordinated to Eu^{3+}. The LOD for PMPA is 7×10^{-3} ng/mL in solution, with a linear range from 1×10^{-2} to 1×10^{4} ng/mL.

MIPs have been considered an advanced technique in biosensing methods for overcoming the drawbacks exhibited by typical antibodies, peptides and enzymes that are commonly used as molecular recognition elements. Zhou et al. [72] reported for the first time an MIP-based potentiometric sensor for MPA employing a surface imprinting technique coupled with a nanoscale transducer, indium tin oxide. However, the accuracy, precision and sensitivity are rather poor, as there is a significant response only in the range of 4.80×10^{3} to 5.95×10^{7} ng/ml of MPA, even though the authors claim an LOD of 4.8×10^{3} ng/mL. Later, Prathish et al. [73] developed a highly sensitive and selective MIP-based potentiometric sensor involving the preparation of MPA imprinted polymer particles in 2-nitrophenyloctyl ether (NPOE), embedding in polyvinyl chloride (PVC) matrix, and removal of the template by Soxhlet extraction. The sensor responds to MPA in the concentration ranges of 4.80 to 9.60×10^{3} ng/mL and 9.60×10^{4} to 9.60×10^{6} ng/mL, with an LOD of 4.80 ng/mL. The MIP-based MPA sensor also did not give false positive readings with some common phosphorus compounds that are most likely to interfere, such as phosphoric acid (H_3PO_4) and sodium dihydrogen phosphate (NaH_2PO_4). Furthermore, the developed sensor was found to be stable for 2 months and could be reused more than 20 times without any loss in sensing ability.

The benefits of nanotechnology make it ideal for sensor development for environmental and biological monitoring. Newman et al. [74] designed a colorimetric sensor based on gold nanoparticles (AuNPs) attached to a planar quartz substrate for detection of MPA. However, the drawback in this work is that it is not able to distinguish between individual organophosphate/phopshonate (OPP) compounds. Therefore, the detection value was based on the presumptive value of OPP compounds present in the samples. Later, Dasary et al. [75] developed an ultrasensitive gold nanoparticle-based surface-enhanced fluorescence (NSEF) spectroscopy technique for screening NA hydrolysis products such as PMPA and MPA. The gold nanoparticles were stabilized or capped by europium (Eu^{3+}) and used as a spectroscopic probe. The detection is based on the fact that the binding constant of Eu^{3+} ions with MPA/PMPA is much higher than that of gold nanoparticles. In the presence of MPA/PMPA, Eu^{3+} ions are released from the gold nanoparticle surface, and thus, a very distinct fluorescence signal change was observed (Fig. 8). The gold nanoparticle-based NSEF had good sensitivity to MPA/PMPA detection, which indicates that the NSEF probe can provide a quantitative measurement.

Bruno et al. [76] reported their first attempt to develop a one-step (bind and detect) fluorescence assay for MPA using a novel competitive fluorescence resonance energy transfer, FRET-aptamer approach. The analyte was first labeled with a known quencher (Q) and then allowed to bind and complex with a fluorophore (F)-labeled aptamer. The aptamer could be singly labeled internally during solid-phase synthesis or multiply labeled internally by asymmetric PCR using F-labeled deoxynucleotides and the aptamer's complementary DNA as a template to produce single-stranded F-labeled aptamers. The F-labeled aptamer-Q-ligand complex was then purified by gel filtration over Sephadex™, and the complex was identified in the fraction or fractions demonstrating the highest simultaneous absorbance for DNA, the analyte, F, and Q at their respective peak absorbance wavelengths. The FRET-aptamer-Q-target complex was then diluted and used in competition with unlabeled target analyte

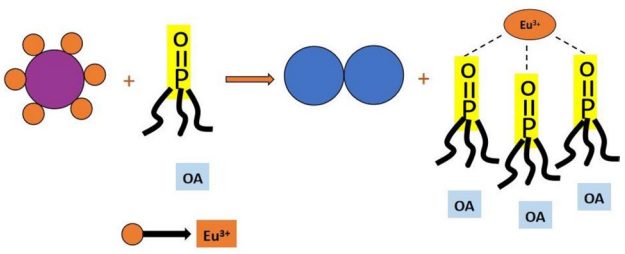

Fig. 8 Schematic representation of a gold nanoparticle-based surface-enhanced fluorescence (NSEF) assay for OA (organophosphorus agent) detection [75]

to develop FRET titration curves. The approach yielded low micrograms per milliliter LODs for MPA with generally low levels of cross-reactivity for unrelated compounds such as acetylcholine (ACh) and bovine serum albumin (BSA).

Chemical sensing and biosensing still have not reached maturity in AMPA/MPA detection, as there have been only a few studies performed 10 years ago. The sensitivity of the sensors was also far lower than that of spectroscopy techniques. The challenge could be the possibility of obtaining stable (bio)sensing molecules because the sensor's efficiency is limited by the stability of the (bio)sensing molecules, such as enzymes and DNA. Biosensor design and construction is a multidisciplinary endeavor and may require expertise from various fields, such as protein engineering, molecular biology, affinity chemistry, nucleic acid molecular dynamics, materials sciences, and nanotechnology. All of the parts of this process have a great diversity of possible configurations that lead to many uncertainties in biosensor design for NA hydrolysis products. Before a sensor gains researcher acceptance, it must be validated by well-established procedures and real samples.

Challenges and future perspectives

Although the use of NAs is prohibited, concerns remain for human exposure to NAs during decommissioning, research, and warfare. Previous investigations on the analysis of NAs have focused not only on the parent compound but also on the hydrolysis products. This is due to the short half-life and low stability of parent compounds in environments containing water. As a result of the rapid hydrolysis of the NA parent compounds, detection of the hydrolysis products is essential.

The success of developing rapid and sensitive analytical methods is dependent on appropriate sample preparation, fast chromatographic separation to achieve symmetrical peaks, and efficient ionization. Efficient sample preparation to avoid severe signal suppression due to the matrix effect is the first key step in achieving high sensitivity and specificity of detection. In general, there are three sample preparation methods for purifying biological samples before injection into an MS/MS analyzer for quantitation of the target analytes, namely SPE, liquid extraction (LLE) or protein precipitation (PPT). LLE is much simpler and less expensive than SPE. However, it is not suitable for hydrophilic metabolites unless derivatization is performed (which is commonly used for GC–MS). Hence, LLE is usually used for the determination of a single analyte but is not suitable for the simultaneous quantification of multiple analytes that have significantly different lipophilicity. PPT is the simplest method of sample pretreatment, as it involves only the addition of a precipitating solvent, subsequent vortex, and centrifugation. The advantages of PPT are that this method is simple, rapid, inexpensive, and suitable for both lipophilic and hydrophilic analytes. In the preparation of biological samples, PPT is widely applied, as it is the simplest sample preparation approach and can be used to quantify both hydrophilic and lipophilic compounds simultaneously, thereby making it more popular than SPE and LLE. The improvements in sample preparation tend to reduce time and solvent consumption. Future trends within sample preparation for analysis include miniaturization and automation and the use of solvent-free techniques to reduce the time required and decrease the possibility of introducing contaminants.

The detection of NA degradation products has been demonstrated with various advanced spectroscopy techniques. However, spectroscopic techniques are time-consuming and expensive and require high-skilled manpower. Rapid detection using sensors has not been fully explored. It is, however, interesting to note that sensor detection applications based on colorimetry utilizing nanomaterials have been investigated but not for compounds involving NA degradation products research. Using nanoparticles such as gold nanoparticles (AuNPs) and silver nanoparticles (AgNPs) for the visual detection of NA hydrolysis products is a highly promising and cost-effective technique. Therefore, the feasibility of using colorimetric-based sensor technology for the detection of NA degradation products should be further explored in the future. Furthermore, enzymatic-based biosensors are also a prominent and favorable technique for the determination of NA hydrolysis products compared to analytical techniques. They are preferable among researchers inventing user-friendly and environmentally friendly devices at lower cost. Future investigations should include increasing the throughput of these methods by achieving faster analysis times. Another interesting pursuit would be reducing LODs and developing a new calibration range to allow detection of these hydrolysis products at extended times postexposure. Additionally, researchers could investigate the applicability of the analysis methods for environmental and food samples, given that the analytes also represent the initial hydrolysis products of the NAs.

In the past 10 years, a number of improvements in the analytical methodologies for the analysis of NA hydrolysis products have been achieved. However, there is no universally accepted analytical method for NA hydrolysis product analysis today. There is a need for a robust, sensitive and valuable method for the determination of a number of representative NA compounds that need to be monitored based on their properties, exposure behavior and possible impact on humans and the environment.

Conclusions

Interest in the analysis and detection of organophosphate NAs and their hydrolysis products (AMPAs/MPA) has increased sharply in the last few years in response to monitoring efforts needed for anti-terrorist activities and to those defined by the Chemical Weapons Convention (CWC). This review article provides an overview of the background of NAs and their hydrolysis products. The analytical techniques that are used for the analysis of hydrolysis products are also discussed. Most analytical methods still rely on chromatographic techniques such as GC and LC. Analyses based on MS are now fairly routine and can be further improved by providing analyses with precise and accurate masses and faster scan speeds that provide further gains in sensitivity. Although these techniques are of high selectivity and sensitivity, most of them are rarely applied to real samples or suffer from destructive real sample analysis. However, in most analyses of NA hydrolysis products, the role of portable and fast sensing technologies has not been well established or investigated extensively. Thus, analysis methods with the advantages of using relatively simple and inexpensive research instrumentation, low consumption of toxic reagents and short sample preparation times are preferable for AMPA/MPA determination. In the future, more on-site detection methods are expected to be established, and the trends may involve the development of sensors or miniature/portable devices. The main challenges in developing these devices will be the specificity, sensitivity, stability, response time, accuracy, precision, and repeatability/reproducibility of the devices. However, optimizing the features of the developed devices is expected to assist first responders in immediate risk assessment and on-site analysis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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