# *Chapter 4*

# *Detection and identification of alkylphosphonic acids by positive electrospray ionization tandem mass spectrometry using tricationic reagent*

## 1. Introduction

In the case of chemical terrorism, there is interest in detecting chemical warfare agents (CWAs) and their hydrolysis products at increasingly lower concentration. It is of utmost importance to minimize casualties, to make available appropriate medical treatment to exposed first responders or victims, and for site remediation purposes. However, retrospective detection and identification of these compounds is also important to support and corroborate the verification program of CWC. Therefore, development of analytical methods for detection and identification of CWAs and their hydrolysis products got impetus to meet perceived challenges. Very high sensitivity of an efficient analytical method is the prerequisite to support the verification program of the CWC and give definitive evidence of chemical terrorism.

As mentioned in chapter 2, in water, nerve agents hydrolyze to non-toxic alkyl alkylphosphonic acids (AAPAs) and subsequently slowly to alkylphosphonic acids (APAs). AAPAs and APAs are significant for the verification of CWC, as they would not be routinely detected in environmental samples, and their detection and identification in a given sample suggests prior presence of nerve agents. The public health sector is interested in detecting trace amounts of AAPAs and APAs as markers of nerve agent exposure. Thus, a reliable and sensitive technique is required to detect these compounds in water samples in order to verify compliance or non-compliance of CWC and for response to chemical terrorism incidents. The most frequently used analytical method for the detection of AAPAs and APAs is based on modern separation technique gas chromatography (GC) coupled to mass spectrometry (MS) [1]. Due to low volatility of AAPAs and APAs, GC-MS is not a suitable technique for their direct analysis, as it requires time consuming derivatization and sample preparation prior to analysis.

Nowadays, liquid chromatography coupled to mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) has become a method of choice to identify CWAs and their degradation products in aqueous samples or extracts without the need for derivatization and sample treatment [2]. In most of the LC-MS or LC-MS/MS methods for the determination of AAPAs and APAs is achieved using electrospray ionization (ESI) and atmospheric chemical ionization (APCI) techniques in combination with liquid chromatography. Read and Black reported that ESI provided better sensitivity compared to APCI [3]. They demonstrated the absolute limit of detection (LOD) in the range of 4-8 ng for APAs. The most recent method by Kolakowski et al, reported the absolute LOD from 2-7 ng for hydrolysis products of CWAs in water samples and different types of food matrices [4]. In addition, LC-MS and LC-MS/MS methods were reported for detection of hydrolysis products of CWAs in environmental and biological matrices [5-15].

As it is capable of detecting the involatile compounds, ESI is the first choice for detecting the involatile degradation products of CWAs. ESI is highly complex process and the response of analyte depends upon the characteristics of the solvents, chemical and physical properties of analyte, concentration of additives and various instrumental parameters [16]. Due to highly polar nature of AAPAs and APAs, their ESI response is very low in comparison to their esters.

For ESI-MS, various methods have been reported for increasing the response of a non-responsive analytes. Derivatization reaction enhances the ESI response of an analyte, either by making it more easily charged or by increasing its surface activity [17]. An alternative approach is to bind a small organic analyte with relatively large molecule such as peptide [18]. This approach significantly increase the response and signal-to-noise ratio for the analyte because the mass of adduct is shifted out of the low-mass region of the mass spectrum – a region that is typically complicated by the presence of solvent clusters and contaminants [17]. Furthermore, ESI sensitivity of analyte can be increased by addition of organic modifiers to mobile phase. Recently, Mawhinney et al reported the enhancement in the response of alkyl methylphosphonic acid by post column addition of organic solvents in negative ESI mode [19]. Generally, negative ESI mode is preferred for organic anions and inorganic acids, but often the LODs are too low and do not allow trace analysis [20-22].

Recently, few methods have been developed to detect low molecular weight inorganic monovalent anions and divalent anions by using a dicationic and tricationic reagent to augment sensitivity in positive ESI mode, and thus eliminating the need of negative ion mode. Martinelango et al increased the sensitivity and selectivity for perchlorate anion by post column addition of dicationic reagent [19, 21]. Remsburg *et al* investigated the effectiveness of the 23 dicationic salts for the formation of gas-phase stable adduct with organic and inorganic anions [22]. Soukup-Hein *et al* used the imidazolium salts for the detection of monovalent and divalent anions [23-24]. This method involves the post column addition of dicationic or tricationic reagents to the mobile phase just before entering the mass spectrometer, leading the formation of stable gas-phase adducts with the an analyte. Adduct still carry a positive charge and can be detected in positive ESI mode with higher sensitivity (Figure 1).

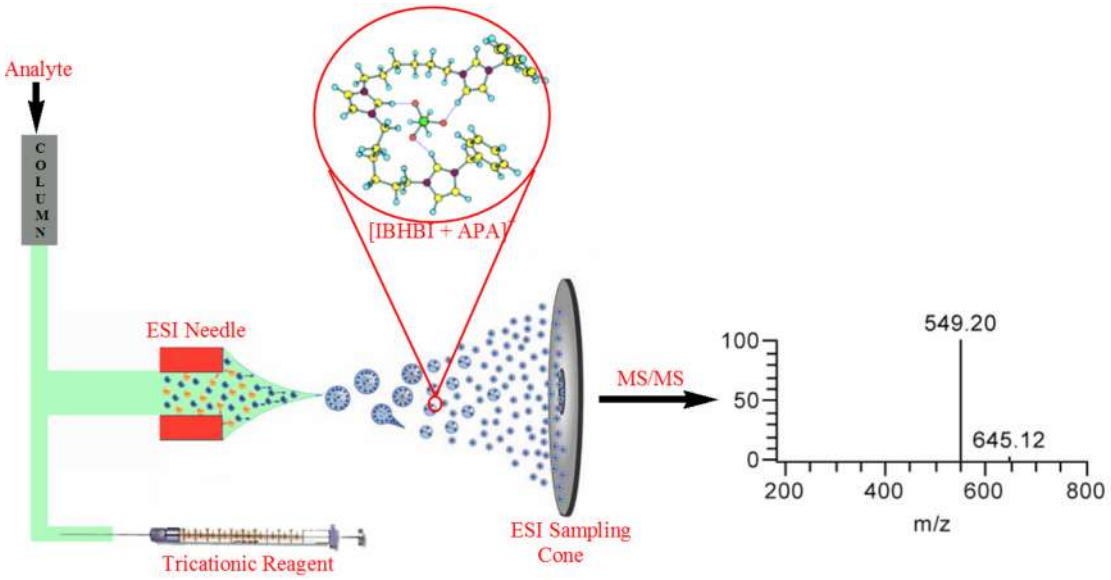


Figure 1. Formation of adduct between tricationic reagent and APA.

There are several advantages to this method: Firstly, dicationic or tricationic reagent forms a stable gas phase adduct with an monovalent anion $[Dication + M]^+$ and divalent anion $[Trication + M]^+$ to give a positively charged complex with higher *m/z* which eliminates the problem of chemical noise near low mass region. Secondly, it also helps in detecting the anions at ultra-trace level without using the complicated sample preparation and derivatization. Moreover, post column addition of dicationic or tricationic reagent not affects the chromatographic separation of analyte.

Thus, by considering the straightforwardness and superior sensitivity of this method, we hereby report development of a LC-ESI-MS/MS method utilizing commercially available solution of imidazolium based tricationic reagent to improve the detectability of APAs in aqueous samples or aqueous extracts.

By using similar principle of signal enhancement, another LC-ESI-MS/MS method has been developed for signal enhancement of AAPAs by utilizing dicationic reagent. The details are given in chapter 5.

## 2. Selection of Analytes

Nerve agent hydrolysis products chosen for the study were methylphosphonic acid (MPA), ethylphosphonic acid (EPA), and *n*-propylphosphonic acid (PrPA) (Figure 2)

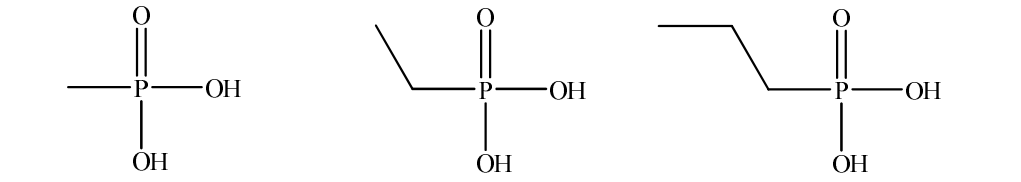


Figure 2. Structures of analytes selected for study

## 3. Result and Discussion

### *2.1 Solvent optimization for MS response*

To obtain maximum response of [IBHBI + APAs]$^+$ adduct in positive ESI mode, the composition of solvent was optimized. Infusion experiments conducted during the method development using MPA as representative of APAs, revealed that [IBHBI + MPA]$^+$ response was maximum with methanol:iso-propanol (80:20 v/v). This enhancement in [IBHBI + MPA]$^+$ response could be due to increased efficiency of gas phase generation of ions caused by low surface tension, higher volatility and less solvation of ions in comparison to pure aqueous solvents [25]. Hence, in further experiments solvent composition for MS analysis was fixed to methanol:iso-propanol (80:20 v/v).

### *2.2 ESI-MS/MS spectrum*

When trication and dianion of APAs were mixed and subjected to positive ESI-MS analysis, the mono-positive adduct [IBHBI + APA]$^+$ was observed. Formation of

gas phase $[IBHBI + APA]^+$ adduct is exemplified with IBHBI and MPA in Fig. 1. $[IBHBI + APA]^+$ adducts for MPA, EPA, and PrPA were detected at *m/z* values of 645, 659, and 673 respectively. The first advantage of this technique was reduction of low-mass region noise by moving anion to higher mass range, e.g. MPA (MW 96) shifted to *m/z* 645. Secondly, detection of anions in positive mode circumvented problems associated with negative mode ESI-MS [24]. ESI-MS/MS spectra of MPA, EPA and PrPA were recorded at 645→549, 659→549 and 673→549 transitions respectively.

In all the cases, MS/MS fragmentation of $[IBHBI + APA]^+$ adducts resulted in the formation of fragment ion *m/z 549*, which is used for the detection of APAs. Thus, in MS/MS experiments, the final ion detected is not the anion of interest but the remnant of the trication. When the $[IBHBI + APA]^+$ adduct is excited and fragmented in MS/MS experiments, $[M-2H]^+$ is the most abundant fragment. This fragmentation is formed from trication by the loss of neutral alkylphosphonic acid; where phosphonate dianion abstracts two most acidic protons from the imidazole rings, as shown in Figure 3.

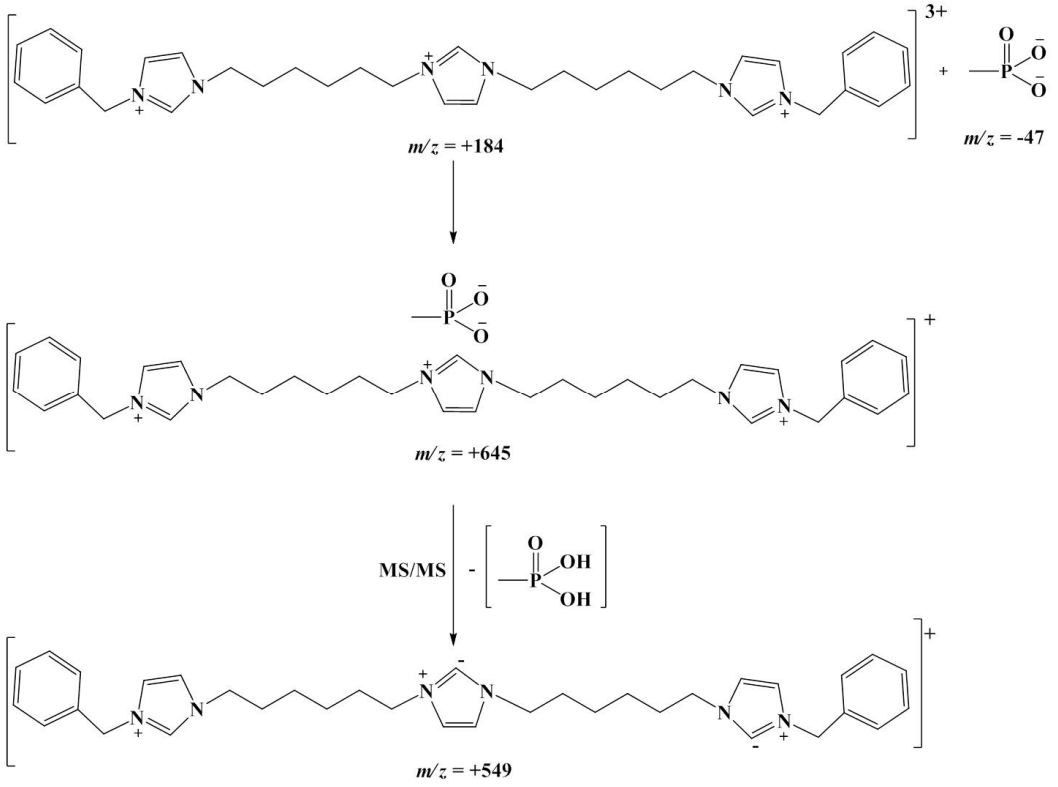


Figure 3. Formation of the detected [IBHBI + MPA]+ adduct and its proposed MS/MS fragmentation pathway.

### *2.3 Concentration of IBHBI*

Adduct formation between tricationic reagent and an analyte depends upon their relative amounts where relatively small concentration of tricationic reagent can form less amount of adduct, the higher concentration of tricationic reagent can add background leading to interferences with analytes. Therefore, optimum amount of IBHBI (that could generate highest signal of APAs in ESI-MS) was optimized by observing the response of $[IBHBI + APA]^+$ as a function of IBHBI concentration. For optimization of IBHBI concentration, 5 µL of 10 µg $mL^{-1}$ solution of MPA was injected through six port loop injection valve. Varying amounts of IBHBI were infused with the syringe pump at a flow rate of 5 µL $min^{-1}$, mobile phase (methanol:isopropanol 80:20) was flown at the rate of 300 µL $min^{-1}$ through LC pump. Molar concentrations of IBHBI were varied from $10^{-4}$ M to 20 x $10^{-4}$ M. Response of $[IBHBI + MPA]^+$ adduct is shown in Figure 4. It is evident that increasing the concentration of IBHBI from $10^{-4}$M to 8 x $10^{-4}$M, also increased the response of adduct, after this the response was constant up to 14 x $10^{-4}$M and began to drop thereafter. Hence, further experiments for all the three APAs were conducted at 10 x $10^{-4}$M concentration of IBHBI. Data in Figure 4 indicate when concentration of IBHBI was increased from $10^{-4}$M to 8 x $10^{-4}$M, there was almost five time increase in response of MPA.

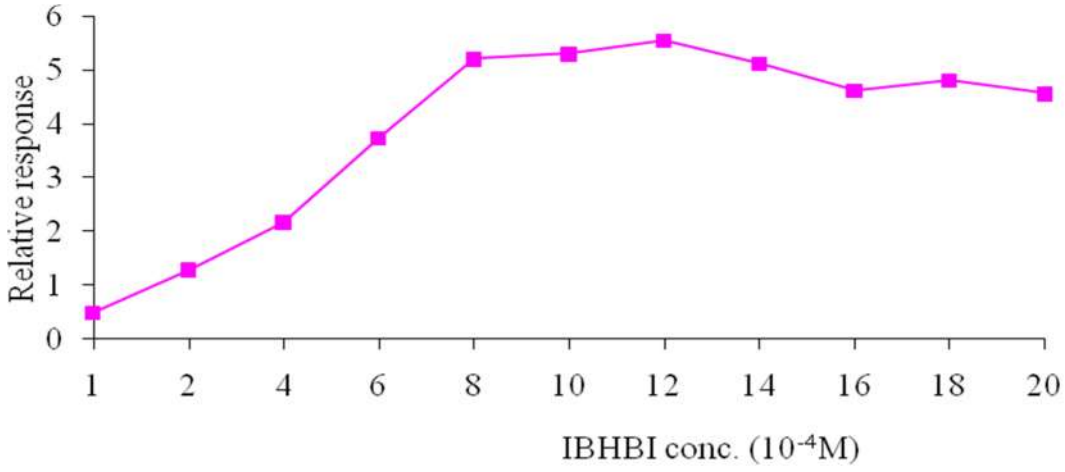


Figure 4. Effect of tricationic concentration on the response of $[IBHBI + MPA]^+$ adduct.

The post column addition of IBHBI solution enhances the response of APAs in the positive ESI-MS/MS mode. This can be explained in the following manner: APAs

are polar and small molecular weight compounds. In the electrospray ionization process, these analyte tend to be more hydrated and reside in the interior of the electrospray droplets, which in turn can lead to poor ionization of analytes, and thus showing the lower response. When IBHBI solution was added to mobile phase (by using Y-shaped tee just before MS), the adduct formation takes place between IBHBI and divalent anion. During the electrospray ionization process, this adduct would prefer to reside at droplet air interface due to its higher hydrophobocity. Consequently, these adducts would enter the gas phase more readily in comparison to hydrated anions (in the absence of tricationic reagent) leading to augment the response.

### *2.4 Effect of pH*

Acid dissociation constants of APAs play an important role in the formation of adduct with tricationic reagent, hence are also supposed to influence the ESI response. APAs are polar and have $pK_{a1} \approx 2.41$ and $pK_{a2} \approx 7.54$ [26]. Thus, at pH more than 9.5, APAs should remain completely in di-anionic form which form adduct with tricationic reagent (IBHBI). To investigate the effect of pH on the response of [IBHBI + APA]$^+$ adduct, each analyte was spiked at a concentration of 0.5 µg mL$^{-1}$ in water having different pH values. pH of water sample was adjusted using hydrochloric acid and ammonium hydroxide. Response of [IBHBI + APA]$^+$ adduct was recorded as a function of pH, and results are summarized in Figure 5.

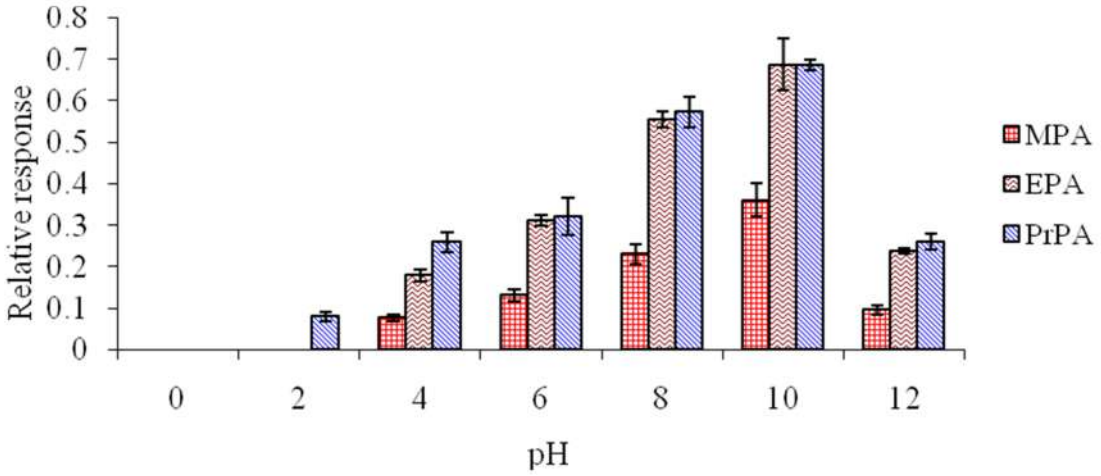


Figure 5. Effect of pH on the responses of [IBHBI + APA]+ adduct.

It is evident from Figure 5 that the response of APAs increased with increase in pH of sample. At pH higher than 9.5, APAs remains completely in di-anionic form which increase the probability of formation of adducts with tricationic reagent (IBHBI). Therefore, for further experiments, the pH of sample was kept to 10. Too basic solution also suppressed the ionization of adduct and/or drop formation in electrospray, causing reduction in response.

### *2.5 Interferences of salts*

Formation of the adduct $[IBHBI + APA]^+$ is a competitive process, and depends upon the total concentration of anions and the concentration of IBHBI. If other anionic species are present in a sample, then their relative affinities towards tricationic reagent determine the response of adducts of APAs. Therefore, it was imperative to investigate the interferences caused by different anions. For this purpose, water samples containing varying amounts of ammonium nitrate, ammonium chloride, ammonium formate, ammonium acetate and ammonium hydroxide were spiked with APAs at 0.5 μg $mL^{-1}$ and internal standard at 2 μg $mL^{-1}$. These samples were subjected to ESI-MS/MS analysis under the optimized conditions. Results of this study are depicted in Figure 6. It is evident from Figure 6 that at lower concentration of salts, there was no significant decrease in the response of $[IBHBI + APA]^+$ adduct. Thus even in the presence of these anions, APAs prefer to bind with tricationic reagent due to their dianionic character and hydrophobicity. However, at higher concentration of salts response of adducts of APAs was decreased which could be explained on the basis of matrix effect. Non-volatile materials can decrease the efficiency of droplet formation through coprecipitation of analytes or preventing droplet from reaching their critical radius required for gas phase ions to be emitted [27].

### *2.6 Development of LC-ESI-MS/MS Conditions*

Having optimized the ESI-MS/MS method for the identification of APAs using IBHBI, next part of the study was focused on conjugating the LC with ESI-MS/MS. In ESI-MS/MS, water samples were introduced directly in mass spectrometer using 5 μL loop and in LC-ESI-MS/MS, samples were introduced in mass spectrometer through LC column using 20 μL loop. To obtain good chromatographic profile of APAs various compositions of mobile phase were

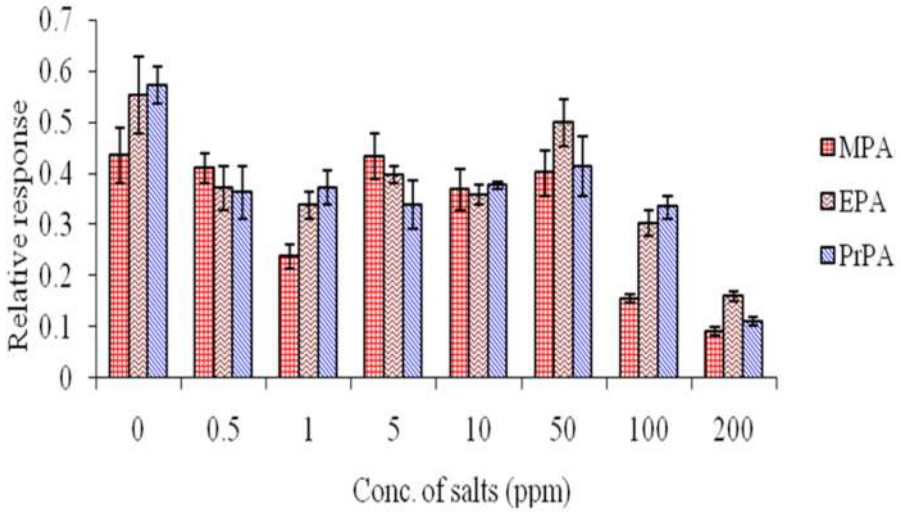


Figure 6. Effect of salt interferences on the responses of [IBHBI + APA]+ adduct.

attempted. Best chromatographic profile was obtained with solvent composition of methanol:water (50:50 v/v). $[IBHBI + APA]^+$ adducts were also detected with this solvent composition without significant compromise with sensitivity and further LC-ESI-MS/MS analysis were performed using this solvent composition as mobile phase.

### *2.7 Performance of analytical method*

To evaluate the performance of the developed method, validation parameters such as limit of detection (LOD) and linear dynamic range (LDR) based on a signal-to-noise ratio of 3 and 10, respectively, were studied. Standard solutions of APAs with varying concentrations were prepared in water, and their pH was adjusted to 10 using ammonium hydroxide. As optimized earlier, in ESI-MS/MS experiments, samples (5 µL) were injected through six port injection loop using methanol: iso-propanol (80:20) as mobile phase and IBHBI solution was ($10 \times 10^{-4}$M) added through syringe pump at a flow rate of 5 µL $min^{-1}$. In LC-ESI-MS/MS analysis, 20 µL of samples were injected through Rheodyne injector using a mobile phase composition of methanol:water (50:50 v/v) at flow rate of 300 µL/min. Other instrument operational parameters were similar as defined in the experimental section. With LC-ESI-MS/MS analysis, a good precision (RSD $\leq$ 7.3%) was obtained in comparison to ESI-MS/MS analysis (RSD $\leq$ 13.9%). Analytical figures of merit are summarized in Table 1. In LC-ESI-MS/MS analysis, linearity ranges were achieved more than 2 orders of magnitude in comparison to single order of magnitude by ESI-

MS/MS analysis (Table 1). The large experimental error and relatively low linearity range in ESI-MS/MS analysis can be attributed to injection volume variations. The absolute detection limits determined with the use of the IBHBI are compared in Table 1. With LC-ESI-MS/MS, all the three APAs were detected at 0.1 ng compared to 0.05 ng by direct ESI-MS/MS. LC-ESI-MS/MS chromatograms obtained for three APAs are depicted in Figure 7. ESI-MS/MS analysis showed better absolute limit of detection for all the three APAs but with relatively less precision.

For APAs, the absolute detection limits determined using IBHBI in positive mode of ESI was compared with absolute detection limits reported in literature. The detection limits achieved using a combination of electrospray ionization and high-field asymmetric

Table 1
Linear dynamic range and limits of detection of [IBHBI + APA]+ adduct.

| | ESI-MS/MS | | | LC-ESI-MS/MS | | |
|---|---|---|---|---|---|---|
| Analyte | Linear dynamic range (ng $mL^{-1}$) | LOD (ng $mL^{-1}$) | Absolute LOD (ng) | Linear dynamic range (ng $mL^{-1}$) | LOD (ng $mL^{-1}$) | Absolute LOD (ng) |
| MPA | 100-1000 | 10 | 0.05 | 10-2000 | 5 | 0.1 |
| EPA | 100-1000 | 10 | 0.05 | 10-2000 | 5 | 0.1 |
| PrPA | 100-1000 | 10 | 0.05 | 10-2000 | 5 | 0.1 |

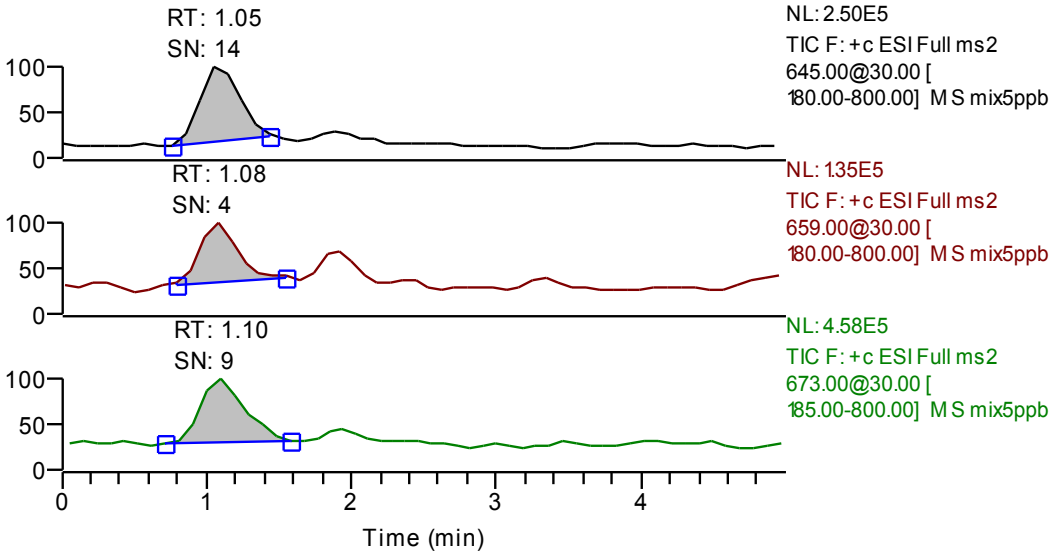


Figure 7. LC-ESI-MS/MS of three APAs on a Sperisorb S5W in a positive ionization mode using IBHBI.(A) MPA (0.1 ng), (B) EPA (0.1 ng), and (C) PrPA (0.1 ng).

waveform ion mobility spectrometry (ESI-FAIMS-MS/MS) were higher (2-7 ng) for the APAs in aqueous samples when compared to this method [5]. Read and Black reported the absolute detection limit range from 4-8 ng for APAs by using LC-APCI-MS/MS [3]. By using a tricationic reagent, the detection limits were improved by at least 1.3 orders of magnitude compared with those reported by other LC-MS/MS methods. These limits of detection are adequate for the determination of APAs in aqueous samples to verify the compliance of CWC. Generally, the spiking level of analytes in proficiency tests conducted by the OPCW ranges from 1-10 μg $mL^{-1}$. Thus, the developed LC-ESI-MS/MS method can be used even if the analytes are present at very low level, the method can also be use in case of allegation of use of CWAs, where even if the concentrations of compound is <1ppm.

### *2.8 Application of method to official proficiency test samples*

To investigate the applicability of the developed LC-ESI-MS/MS method, water samples obtained from OPCW during 22$^{nd}$ and 24$^{th}$ official proficiency test (OPT) were tested and results obtained are summarized in Table 2. Original water samples (100 μL) were taken and further diluted to 10 times with triple distilled water and their pH was adjusted to 10 using aqueous solution of ammonium hydroxide. Fig. 5 shows LC-ESI-MS/MS chromatogram of 10 times diluted sample and quantitative results are shown in Table 2. We determined the concentration of MPA, EPA and PrPA in water samples using optimized LC-ESI-MS/MS conditions. The concentration of MPA, EPA and PrPA were found to be 1.1 μg $mL^{-1}$, 9.4 μg $mL^{-1}$ and 9.5 μg $mL^{-1}$ respectively as shown in Table 2. The spiking concentration of MPA and

Table 2
Quantification of APAs in OPT water sample.

| | | | Concentration (μg $mL^{-1}$) | |
|---|---|---|---|---|
| Analyte | Equation | $R^2$ | Spiked | Determined |
| MPA[a] | $y = (4 \times 10^{-5})x + 0.003$ | 0.994 | 2 | 1.1(±0.17) |
| EPA[a] | $y = (4 \times 10^{-5})x + 0.003$ | 0.998 | 10 | 9.4 (±0.25) |
| PrPA[b] | $y = (7 \times 10^{-5})x + 0.002$ | 0.996 | 10 | 9.5 (±0.70) |

[a] 22$^{nd}$ OPT water sample

[b] 24$^{th}$ OPT water sample

EPA in 22nd OPT water sample were 2 µg $mL^{-1}$ and 10 µg $mL^{-1}$ respectively with background of polyethylene glycol 200, polyethylene glycol 250 and polyethylene glycol 300 at the concentration of 150 µg $mL^{-1}$ each along with anhydrous calcium chloride and 3,3-dimethyl-1-butanol at 250 and 10 µg $mL^{-1}$, respectively [28]. The spiking concentration of PrPA in 24th OPT water sample was 10 µg $mL^{-1}$. 24th OPT water sample also constituted the following background chemicals; magnesium sulfate heptahydrate, 120 µg $mL^{-1}$; sodium sulfate anhydrous, 284 µg $mL^{-1}$; calcium chloride dehydrate, 222 µg $mL^{-1}$; dichlomethane, 2.5 µg $mL^{-1}$; polyethylene glycol 200, 505.4 µg $mL^{-1}$; sodium carbonate anhydrous 106 µg $mL^{-1}$; polyethylene glycol 400, 505.4 µg $mL^{-1}$; 2,3-dihydroxytoluene, 10.05 µg $mL^{-1}$; and neodymium(III) hydroxide, 15.6 µg $mL^{-1}$ [29]. Relatively low recovery of MPA over EPA and PrPA could be attributed to its 5 times low spiking concentration, due to which the matrix effects were more pronounced for MPA in comparison to EPA and PrPA leading to exhibit lower recovery of MPA. Good correlation of spiked and determined concentration of MPA, EPA and PrPA in OPT samples even in the presence of various background chemicals demonstrates the functioning of the developed LC-ESI-MS/MS method. Figure 8 clearly demonstrate that applicability of the developed LC-ESI-MS/MS method for the determinations of APAs in water samples without any sample treatment. Although spiking of APAs was high in OPT water samples, the developed LC-ESI-MS/MS method can be used without any sample treatment for the detection of APAs in aqueous sample or aqueous extracts.

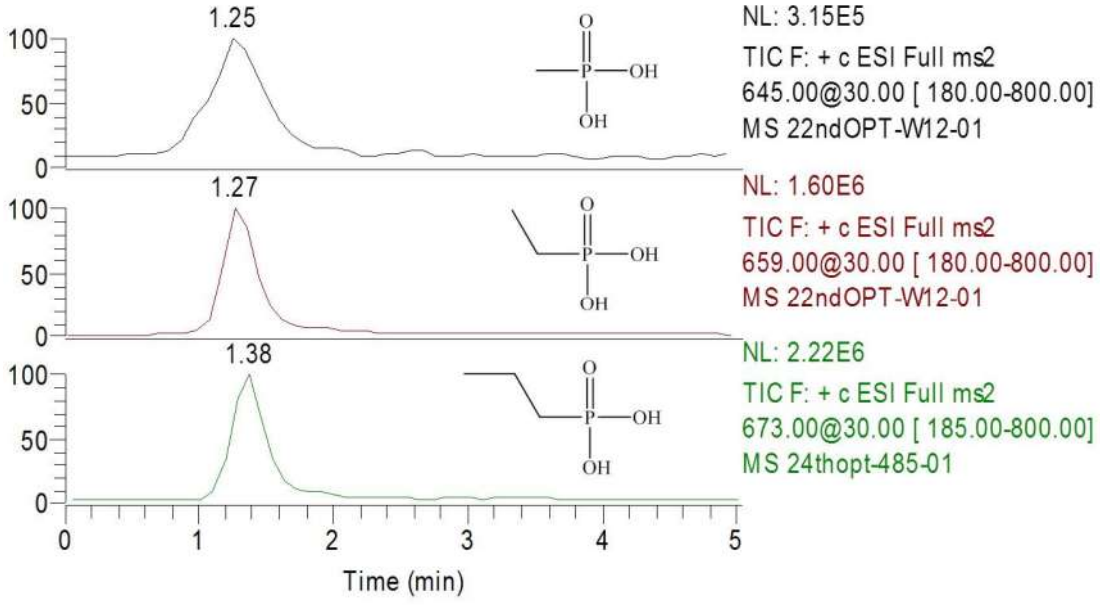


Figure 8. LC-ESI-MS/MS chromatogram of 10 times diluted OPT water sample.

## 3. Experimental

### *3.1 Chemicals and samples*

APAs compounds were synthesized in our laboratory as per reported procedure [30] and were more than 99% pure by $^{31}P$ NMR and $^{1}H$ NMR analysis. Stock standard solutions of each phosphonic acid were prepared in acetonitrile at a concentration of 10 mg $mL^{-1}$. A working composite standard solution was prepared by combining an aliquot of each of the stock solution and diluting the mixture with triple distilled water. The concentration of analytes was kept 500 ng $mL^{-1}$ unless otherwise mentioned. 2 μg $mL^{-1}$ of tributyl phosphate was used as an internal standard. 1,3-Imidazolium-bis-(1-hexyl-benzyl-imidazolium)-trifluoride (IBHBI) solution (0.005M) in water: methanol (1:1), ammonium formate, ammonium acetate and tributyl phosphate were purchased from Sigma-Aldrich (New Delhi, India). Hydrochloric acid, ammonium nitrate, ammonium chloride and ammonium hydroxide (25% ammonia solution) were obtained from Merck (Mumbai, India). MS grade methanol, water, acetonitrile and iso-propanol were obtained from Sigma-Aldrich (New Delhi, India). All the solvents and solutions were filtered through 0.45 μm nylon filters (Millipore, New Delhi, India).

### 3.2 *ESI-MS analysis*

Mass spectral analyses were performed with a LCQ Advantage ion trap spectrometer (Thermo Electron Corporation, San Jose, CA, USA) with a six port injection valve used to make injection. Except as stated, all analyses were done by loop injections. Samples were directly introduced into the mass spectrometer using a 5 μL sample loop. A flow rate of 300 μL $min^{-1}$ was provided by a Surveyor HPLC pump (Thermo Electron Corporation, San Jose, CA, USA). IBHBI solution was introduced into the sample stream at a flow rate 5 μL via a Y-shaped mixing tee using a Thermo syringe pump. The ESI source parameters were as follows: voltage, 6.0 kV; capillary temperature, 350°C; capillary voltage, 4.5 V; tube lens offset, 20.00 V; sheath gas, 31 arbitrary units of pressure. The system was operated in positive ion mode. Helium was continually flowing into the collision cell at a pressure of 0.1 Pa ($10^{-3}$ Torr) during the ESI-MS/MS operation. Normalized collision energy for MS/MS experiments was set at 30 and the activation time 30 ms. The ESI-MS/MS data were

acquired over the mass range of *m/z* 178-800 . The conditions reported here were optimized for MPA and applied for EPA and PrPA.

### *3.3 Liquid chromatography conditions*

For chromatographic experiments, above configuration of instrument was slightly modified. Chromatographic experiments were carried out with Thermo Electron HPLC with surveyor LC pump (San Jose, CA, USA) fitted with a 20 µL injection loop for sample introduction. A Waters (Bangalore, India) Sperisorb S5W, 2.1 mm x 150 mm microbore column was used for chromatographic analysis. The following solvent composition was made for sample introduction: solvent A (methanol) and solvent B (water). Chromatographic analysis was performed using isocratic elution with 50% A and 50% B at a flow rate of 300 µL $min^{-1}$. Column temperature was kept at 25 °C.

## 4. Conclusions

A highly sensitive LC-ESI-MS/MS method was developed for the detection and identification of anionic degradation products of nerve agents in aqueous samples using a commercially available solution of IBHBI; which demonstrated the absolute limit of detection for APAs at 0.1 ng. A gas phase association between IBHBI and dianion of APAs eliminates the problem of chemical noise near low mass region and it also helps in detecting the anions at trace level without using the complicated sample preparation and derivatization. Thus, the developed LC-ESI-MS/MS method can be used for the trace level detection of analytes.

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