Post Analysis of Proficiency Test Samples Using Nuclear Magnetic Resonance Spectroscopy

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Abstract

Paper describes the Post Nuclear Magnetic Resonance Spectroscopic analysis of proficiency test samples using Phosphorus NMR. In our study two-dimensional ^{1}H-^{31}P NMR method was optimized effectively for identification of Chemical weapon convention related scheduled organophosphorus compounds in the environmental samples provided by OPCW during proficiency test, while second report is on post analysis of proficiency test sample, carried out to reexamine the samples to observe the missed organophosphorus compounds and to investigate the causes of missing of organophosphorus chemicals during analysis.

Subject Areas

Analytical Chemistry, Environmental Chemistry

Keywords

Phosphorus NMR, Organization for Prohibition of Chemical Weapons, 2-Dimensional Nuclear Magnetic Resonance, Chemical Weapon Convention, Official Interlaboratory Proficiency Test

1. Introduction

Under CWC (Chemical Weapon Convention) treaty, entered into the force on 29th April 1997, an international organization, "Organization for the Prohibition of Chemical Weapons" (OPCW) ensures implementation of CWC through a stringent verification regime. Till now OPCW has 193 member states [1]-[9]. In order to test the ability of laboratories and to designate the laboratories, OPCW conducts Inter laboratory "Official Proficiency Tests" (OPTs) twice in a year, to rule out any ambiguity during final testing in real scenario, to prove alleged use of CW agents. The designated laboratories have to maintain consistency in the

OPTs to maintain their designation status. Approximately forty to forty-five laboratories from various countries have been participating regularly in these tests [5]-[14].

The most frequently used analytical techniques in OPTs, for the unambiguous identification of CWAs (Chemical Warfare Agents), their precursors and degradation products are, based on Nuclear magnetic resonance (NMR) spectroscopy, Gas Chromatography (GC) in combination with Mass Spectrometry (GC-MS) and/or tandem mass spectrometry (GC-MS/MS), Liquid Chromatography (LC) coupled with MS, and Infrared spectroscopy [4]-[9] [15]-[22]. GC-MS and Nuclear magnetic resonance (NMR) spectroscopy are the most frequently used techniques for analysis and identification of spiked chemicals in environmental samples as per evaluation reports of Official Proficiency Tests [1] [2] [3] [4] [21]-[32].

Since the CWC came into force, many efforts have been made to develop and improve methods for the detection and identification of CWAs, precursors and their degradation products for successful participation in proficiency tests [1] [2] [3] [9]-[33]. The successful participation in OPTs mainly depends on the availability of in-house spectral database of CWC related chemicals, efficient sample preparation, analytical skills of chemists, correct judgment and reporting skills and synthetic expertise [9] [10] [11] [12] [13] [15] [16] [17] [22]-[27] [31].

Nuclear magnetic resonance (NMR) spectroscopy is a valuable analytical technique for the identification of chemicals related to the CWC [32] [35]. More than 75% of the Scheduled chemicals are organophosphorus (OP) compounds, which are conveniently screened by ^{31}P{^{1}H} NMR experiment [9] [14]-[20] [32] [33] [34] [35] in mixtures. NMR gives complete information about the skeleton of the molecules by applying different NMR techniques. One-dimensional (1-D) proton, fluorine, and phosphorus NMR spectra (^{1}H, ^{19}F, ^{31}P{^{1}H}, and ^{31}P spectra) are frequently recorded for analysis of test samples [22]-[35]. Chemicals are identified on the basis of matching of finger print spectra and chemical shifts of samples with spectra of reference chemicals. Normally 2-D NMR techniques are more suitable after screening of mixtures by 1-D NMR for complete structure elucidation of unknown chemicals by showing two and three bonds connectivity of phosphorus with alkyl groups [31]-[40]. Sometimes low concentration of chemical of interest in the presence of huge background may cause problems in NMR analysis. However, the structural specificity of NMR spectra, the capability for the observation of different nuclei, and the 2-D correlation experiments make NMR spectroscopy a valuable technique to be used in combination with other techniques for unambiguous identification of spiked chemicals listed in the Schedules of the CWC [1] [2] [3] [32]-[37]. The ^{31}P{^{1}H} and ^{19}F NMR experiments are more useful than ^{1}H and used as screening tool to check the presence of organophosphorus (OP) compounds [32]-[37]. In some cases where concentration is sufficient the proton-coupled ^{31}P spectra, reveals more detailed structural information by showing splitting patterns, but use of this experiment

is limited due to poor sensitivity [41]. Normally, the low sensitivity of NMR experiments are compensated by concentrating the samples and by running long-term experiments in OPTs (Official Proficiency Tests). In literature number of papers reveals successful use of ^{31}P NMR for analysis of OPTs samples [9] [20] [22] [23] [24] [31] [32] [33] [34] [41] [42]. Review on use of NMR techniques for toxic organophosphorus compound profiling, presents versatility of Nuclear Magnetic Resonance (NMR) spectroscopy in the analysis of toxic organophosphorus (OP) compounds [42]. Two-dimensional [1]H-[31]P Fast-HMQC (Heteronuclear multiple quantum correlation) was optimized for rapid detection of OP compounds at low concentration [34]. The advantage of NMR detection is that it is free from any extra sample preparation, memory effect and cross contamination [32]-[40]. Therefore in this paper we emphasized the importance of NMR based techniques and investigated the possible reasons of missing the compounds during test in water and organic sample of 19th proficiency test using NMR only and performed the optimization of 2D NMR technique using vacuum pump oil sample of 17th proficiency test sample. The effect of concentration and pH on phosphorus chemical shift is also observed. It is also realized that when molecules are having more than two stereogenic centers then identification in NMR becomes difficult at low concentration. During analysis of 19th proficiency test samples we missed OP compounds. Therefore it was planned to reexamine the samples after the test, to find out the reason of failure. The experience gained from the exercises could be useful in decipher the structure of unknown OP compounds during OPTs and helpful in obtaining the required designation.

2. Experimental Part

Two 400-MHz Bruker, Avance and DPX NMR spectrometers were used for the detection of Scheduled chemicals in environmental samples. Temperature was kept 296 to 300 K in all the experiments. Computer operating systems UNIX, Xwin NMR 2.3 and Top spin 1.3 were used. The direct observation BBO probe head (broad band observe) was used for 1-D and 2-D HSQC experiments on ^{1}H and ^{31}P{^{1}H} nuclei with proton broadband (composite pulse) decoupling. ^{19}F{^{1}H} Nucleus was observed by the use of Fluorine selective 5 mm probe head using zgpg pulse sequence, sweep width 37,664.78 Hz, acquisition time 3.48 sec, delay 1 sec., NMR frequency 376.474 MHz, and pulse angle 9.40 μsec. For ^{31}P nucleus, sweep width 43,859.64 swh, acquisition time 2.98 sec, delay 2 sec, td & si 128 K and Magnet frequency was tuned at 161.9755 MHz. ^{31}P{^{1}H}, ^{1}H and ^{19}F {^{1}H}NMR spectra were recorded keeping range −150 to 150 ppm, −0.5 to 14 ppm and −100 to 20 ppm respectively. FID (Free Induction Decay) was Fourier transformed after application of an exponential window function. Baseline correction was done to improve the resolution and signal height of very small intensity resonances.

The sensitivity, resolution checking and pulse calibration was carried out for each nucleus separately. Separate tuning of the probe head for each sample and

nucleus was adjusted to obtain the optimum sensitivity. Accurate homogeneity adjustment was done by doing shimming, in order to achieve the narrowest lines and pure line shape. Spectroscopic grade E-Merck solvents; Deuterium oxide (D_2O), Chloroform-d1 ($CDCl_3$), of >99.9% deuteration were used for NMR samples analysis. Chemical shift (δ) references for calibration of the spectrum scale were used externally 85% H_3PO_4 at 0.00 ppm for ^{31}P, CF_3COOH for ^{19}F at −76.50 ppm. An internal chemical shift reference for ^1H, tetramethylsilane TMS in $CDCl_3$ and 3-(trimethylsilyl) −3, 3, 2, 2-tetradeuteropropionic acid sodium salt (TSPA-d4) in D_2O at 0.00 ppm was used. 5 mm Wilmad Royal Imperial NMR tubes were used for analysis and Tubes were cleaned repeatedly with distilled water followed by acetone, and dried in oven (at 1000°C). Initially samples were analyzed as such without doing any extraction, to check the presence of any volatile compounds by NMR. Sample preparation and analysis were done as per recommended procedure (ROP) of OPCW for test [43].

Three samples (Vacuum pump oil, Organic waste and Soil) along with their corresponding blank samples were received for analysis with proper laboratory coding in Seventeenth OPCW Proficiency Test. In order to analyze the vacuum pump oil sample, 2 ml of sample was extracted with methanol (3 × 800 µl), methanol was evaporated by Nitrogen purging up to dryness in one sample and 700 µl D_2O was added while in second sample evaporation was done up to 400 µl and 300 µl D_2O was added for $^{31}P\{^1H\}$ NMR analysis. This was done to observe the volatile chemicals in samples, but in both the cases same results were obtained. The organic waste sample (500 µl) was taken as such in a NMR tube and 200 µl $CDCl_3$ was added to screen the presence of organophosphorus (OP) compound by $^{31}P\{^1H\}$. Organic sample of 19th OPTs was prepared in the similar manner. The soil sample (500 mg) was extracted with 3 × 500 µl methanol and extracts were combined and centrifuged as per ROP (Recommended Operating Procedure) [33]. The methanol was removed by nitrogen stream until the volume of 200 µl remains. It was make-up up to 700 µl by addition of 500 µl D_2O and analyzed by $^{31}P\{^1H\}$ NMR. The same treatment was given to their corresponding three blank samples. In the nineteenth OPTs, OPCW forwarded three samples; one organic liquid and two water samples. The spiking level of all the chemicals was 6 - 20 µg/ml as per scenario provided by the OPCW. All the samples were stored in deep freezer for two hours so that volatile compound, should get condensed in the sample.

Both the water samples and corresponding blank samples, 500 µl were taken in a NMR tube and mixed with 200 µl D_2O and shaken for one minute on vortex shaker for homogenous mixing.

3. Results and Discussion

Here post NMR analysis of samples of seventeenth and nineteenth OPCW Proficiency tests are being described in detail. In the vacuum pump oil sample of 17th OPTs only one OP Compound, Dimethyl methylphosphonate (DMMP) was found and 2D NMR method was optimized for its trace level detection. While in

Organic sample of 19th OPTs only three OP compounds 4-methylpentyl methylphosphonofluoridate (correctly identified), Pinacolyl methylphosphonic acid (wrongly reported), tetraethyl ethylenediphosphate (false identification) out of four were identified and one compound failed to identify. One OP Compound in Water sample 1, also remained unanswered while other three isomers of O-hexyl methylphosphonate were identified correctly. In this paper, in post analysis we concentrated only on three samples; vacuum pump oil (17th test), organic and water 1 samples (19th test).

3.1. NMR Results of Seventeenth Proficiency Test (Vacuum Pump Oil)

$^{31}P\{^1H\}$ NMR analysis of sample showed no phosphorus signal in soil and organic samples except one at δ 38.401 ppm (**Figure 1**) in vacuum pump oil sample. The concentrated extracted sample of vacuum pump oil was analyzed by 2D NMR technique for assigning the complete skeleton of the compound. Finally, the structure of spiked compound was assigned as dimethyl methylphosphonate (DMMP) with the help of 2D NMR analysis. The methyl groups of CH_3-P=O and CH_3O-P=O groups of DMMP showed connectivity with phosphorus signal appeared at 38.40 ppm with doublet signals at 1.49 ppm ($^2J_{P-H}$ = 17.56 Hz) and at 3.61 ppm ($^3J_{P-H}$ = 10.84 Hz) respectively of ^1H NMR which is shown in **Figure 2**, 2D ^1H-$^{31}P\{^1H\}$ HSQC (Heteronuclear Single Quantum Correlation) spectrum. 2D HSQC NMR of reference sample (**Figure 4**) along with 1D ^1H and $^{31}P\{^1H\}$ is shown in **Figure 3** and matched with spectra of spiked sample (**Figure 2**).

3.2. NMR Results of Nineteenth OPCW Official Proficiency Test

Organic Sample

Eight signals were observed in the range of 33.107 to −18.040 ppm in organic sample after the accumulation of 17,720 scans (**Figure 5**). Three signals out of eight appearing at 0.891 ppm, −1.771 ppm and −18.040 ppm may arose from phosphate type of compounds as phosphates appear in this range in ^{31}P NMR and later reconfirmed by Mass Spectrometry. Since the phosphates are not relevant to CWC, these are not discussed here. The remaining five signals appeared in the range of CWC related compounds.
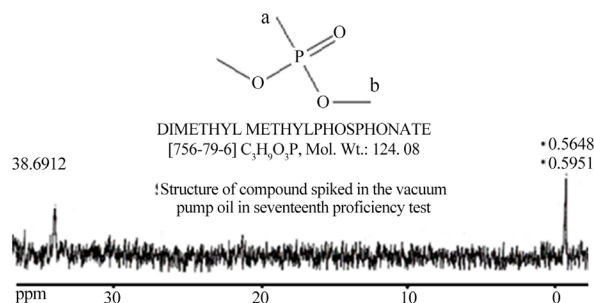

Figure 1. 1D $^{31}P\{^1H\}$ NMR Spectrum of Vacuum pump oil Sample in D_2O Solvent.
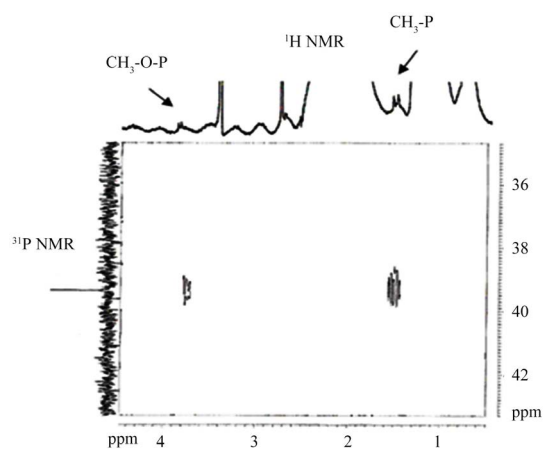
Figure 2. 2D-HSQC ^{1}H -^{31}P NMR Spectrum of oil Sample showing cross peaks for Dimethyl methylphosphonate (DMMP).
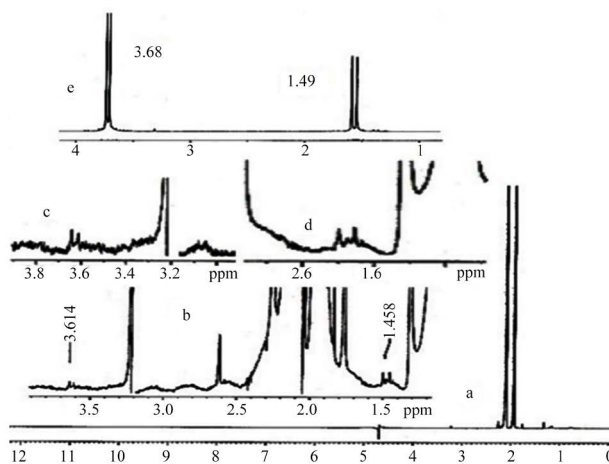

Figure 3. ^{1}H Spectrum of Vacuum pump oil Sample with water Suppression (a), expanded region of ^{1}H Spectrum showing 1 ppm to 4 ppm range of Chemical Shift (b). Expanded region of Spectrum showing doublet of CH$_3$O-P group at 3.61 ppm (c), and of CH$_3$-P group at 1.4 ppm (d). ^{1}H NMR Spectrum of Reference Compound Showing doublets for CH$_3$O-P and CH$_3$-P groups of Dimethyl methylphosphonate (e).
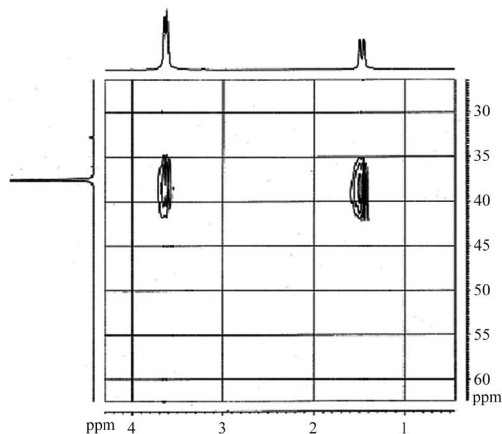

Figure 4. 2D-HSQC ^{1}H-^{31}P NMR Spectrum of Reference Chemical Showing cross peaks for Dimethyl methylphosphonate.
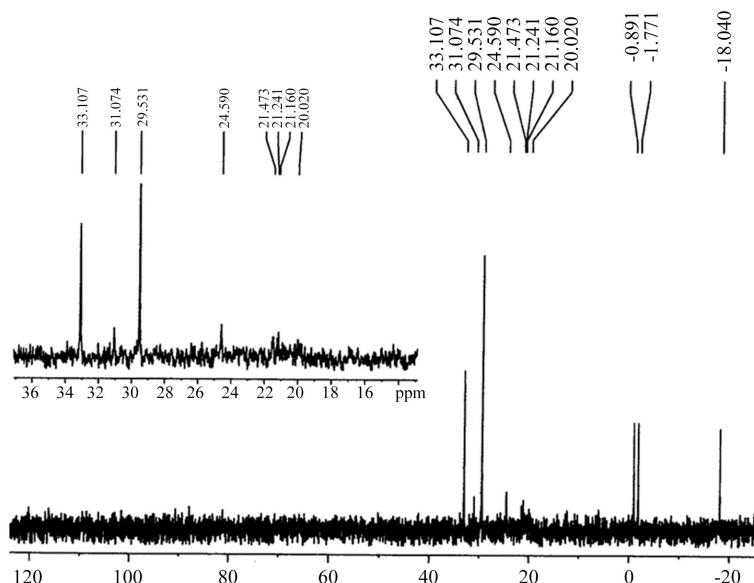
Figure 5. $^{31}P\{^{1}H\}$ NMR Spectrum of Organic Liquid sample with expansion in CDCl$_3$ showing Phosphorus Signals ranging from 33.10 ppm to −18 ppm.

Among the five signals, (33.107, 31.074, 29.531, 24.590, and multiple signals in the range of 21.473 to 20.020), two intense signals were of equal height, and remaining three were having good intensity. The two intense signals at 31.107 and 24.590 ppm were accounted as fluoridate doublet. In order to check our assumption, the δ difference between all the peaks was calculated in Hz. The difference of 1050 Hz was found only for two signals appeared at 31.074 ppm and 24.590 ppm, which is in the range of coupling value of phosphorus with fluorine. The same sample was analyzed by ^{19}F NMR to confirm the presence of fluorine containing organophosphorus compound. It showed two signals at −59.28 and −62.06 ppm due to coupling of fluorine with phosphorus (**Figure 6**). There is pronounced effect of directly attached alkyl group to phosphorus, on P-F coupling constant in fluorine containing OP compounds. The coupling constant for groups CH$_3$-P(O)-F, C$_2$H$_5$-P(O)-F, isopropyl-P(O)-F and n-propyl-P(O)-F is ~1048 Hz, ~1070 Hz, ~1087 Hz and ~1067 Hz respectively. From this data it was inferred that the alkyl group directly attached to phosphorus is methyl ($^{1}J_{P-F}$ = 1050 Hz) (on the basis of P-F coupling constant) which is mandatory for reporting also. So prediction of attached alkyl group to phosphorus could be made on the basis of coupling constant value (J_{P-F}) values, in fluoridates. 4-methylpentyl group of this OP compound confirmed by Mass spectrometry. The structure of this compounds was therefore, assigned as 4-methylpentyl methylphosphonofluoridate after perfect matching with reference sample (**Figure 6**) under the identical experimental conditions. ^{19}F and $^{31}P\{^{/}H\}$ NMR of 4-methylpentyl methylphosphonofluoridate presenting matching of chemical shift values with reference chemical, is shown in **Figure 6**. One compound at 29.531 ppm was identified as Pinacolyl methylphosphonic acid with the help of Mass and phosphorus signal δ, matched with reference at 29.531 ppm.
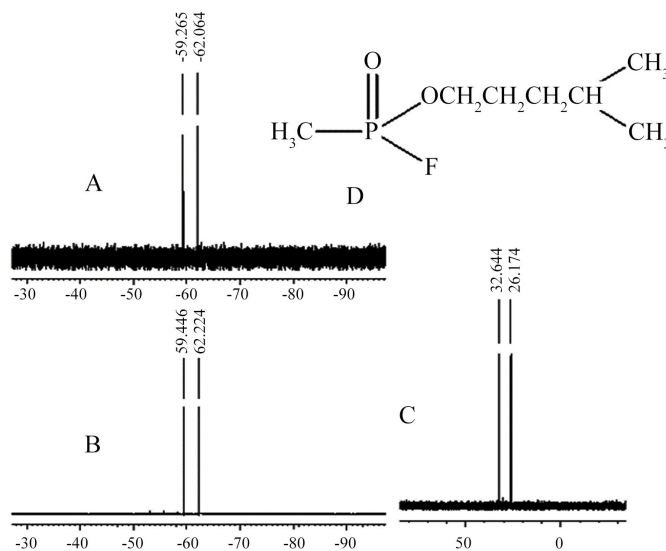
Figure 6. $^{19}F\{^1H\}$ NMR Spectra of Organic Sample (A), Reference Sample (B), showing matching of Chemical shifts. $^{31}P\{^1H\}$ Spectrum of Reference Sample (C), and Structure of Spiked Chemical 4-methylpentyl methylphosphonofluoridate (D).

The multiple signals ranging from 21 to 20 ppm were assumed that these may be because of several diastereomers of one of the spiked compounds or due to presence of more than one compound or degraded product of any OP compound. No NMR and Mass data of this chemical was available in any of the spectral library of OCAD or NIST MS-library [44] and in-housed developed NMR library available with us at that time, hence this compound could not be identified. Hence multiple signals in the range 21 to 20 ppm remained unanswered (Figure 5). Later this molecule was reported as Dipinacolyl dimethylpyrophosphonate by OPCW [45]. So in this test one compound was missed in reporting and one compound was reported wrongly at 33.107 ppm as tetraethyl ethylenediphosphate. OPCW later reported [45] this molecule as new cyclic phosphonate O,O-1,3-trimethylene isopropylphosphonate.

Water sample 1

The pH of the water sample (actual and blank) was found neutral and it was screened as such without any sample preparation, by $^{31}P\{^1H\}$ NMR along with blank sample, in order to avoid the loss of volatile compounds. Results showed that there is no signal in the blank sample. However, the water sample showed four signals at 26.607 ppm, 25.289 ppm, 25.138 ppm and 20.88 ppm in ^{31}P NMR $\{^1H\}$ analysis after 11,080 scans as shown in the Figure 7(A). The chemical shift values of these four compounds (26.607 ppm, 25.289 ppm, 25.138 ppm and 20.88 ppm) showed that these compounds have P=O functionality (as P=O containing OP compounds fall in this range), which might have arisen either from alkyl phosphonic acids or from O-alkyl alkylphosphonic acids. Esters of phosphorus related to scheduled chemicals do not fall in this range in water. These show chemical shift values around 32 to 38 ppm in water. In order to confirm whether these signals have arisen from monoacids or diacids; sample was derivatised
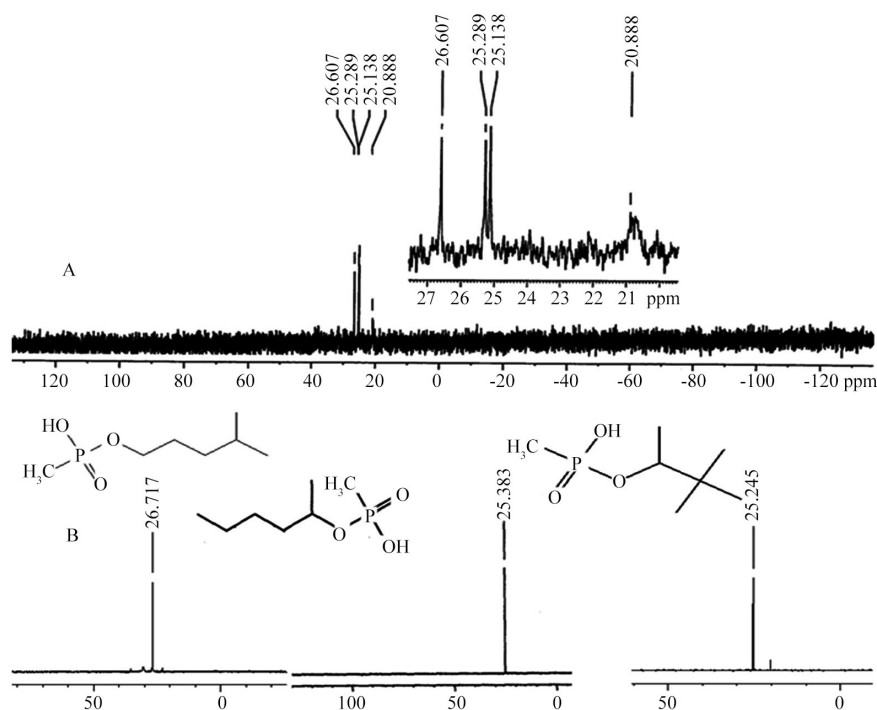
Figure 7. $^{31}P\{^1H\}$ NMR Spectra obtained from analysis of: Water 1 Sample (A), Reference Chemicals (B), in D_2O.

with diazomethane to make their methyl derivatives. The derivatised sample was analyzed by NMR followed by GC/MS and confirmed presence of monoacids. The nature of P-C linkage is confirmed by 2-D NMR experiment. The result of 2D HSQC ^1H-$^{31}P\{^1H\}$) NMR analysis showed connectivity of three ^{31}P signals (range 25 to 26 ppm) with methyl doublet signal in ^1H NMR at ~1.50 ppm, ~$^2J_{P-H}$ = 16 Hz, for three compounds and confirmed that these three chemicals have -CH_3 group attached to phosphorus atom in their structure directly and later these were found isomers as the molecular weight for all the three compounds was same, in Mass analysis (~180 mw). Thus it was confirmed that all the three compounds have the structure of alkyl methylphosphonic acids. Furthermore, by getting the clue of molecular weight of the compounds from GC/MS, it was easy to assign the P-OR group of the structures and it was assigned as O-hexyl group. Therefore, to assign the chain of P-OC$_6$H$_{13}$ group in their structures various derivatives of hexyl methylphosphonic acid were synthesized by using different isomers of n-hexanol. The pH in all the analysis was kept neutral. Thus, they were identified as positional isomers of hexyl methylphosphonate after matching their signature with synthesized reference samples. Probably, because of the resemblance in the structures of positional isomers their chemical shift values were found very close to each other. However, the change in concentration and pH were critical factors for matching their spectra with reference samples. In order to confirm the skeleton of spiked compounds, 2D HSQC NMR experiment was performed with the concentrated sample and results reconfirmed the presence of both P-CH$_3$, P-OCH$_2$, and P-OCH groups. After, assign-

ing the structures for the three signals it was necessary to have some meaningful conclusion about the fourth signal of 20.88 ppm. But we were failed in identifying this compound. This was assumed that this hump may be due to methylphosphonic acid (MPA). But chemical shift value of this signal did not match with any of the spectral data of MPA available in library at neutral pH. Water 1 sample was analyzed in $CDCl_3$ also, after evaporation of water, to analyse the hump signal of 20 ppm (Figure 7(A)), with the idea that due to less solubility chemical is not coming properly in water solvent, but due to low concentration during extraction, it was totally removed from the mixture and no signal was observed for this chemical in organic medium (only three signals found). Then it was also assumed that this hump is not due to OP chemical, some artifact is present in the sample or it may be the result of bad shimming or some field variation in long acquisition time. Unfortunately, later, it was interpreted as signal of methylphosphonic acid in posttest analysis.

Assignment of closely appeared signals at δ 26.607, 25.289 and 25.138 ppm

The assignment of closely lying signals of OP compounds was done by standard addition method (adding reference compounds in mixture one by one). First 10 μl sample of each reference compound (three isomers of hexyl methylphosphonic acid) was taken in the NMR tubes separately. Each sample was prepared by addition of 650 μl D_2O and pH maintained neutral. Their ^{31}P NMR spectra were recorded and chemical shift values were observed, they were all showing different ppm values as shown in above heading and closely related with the spiked sample. As we know that there is an effect of pH, concentration and presence of other chemical on the chemical shift and this may cause variation of ~1 to 2 ppm; to study the effect of interference on the chemical shift value, addition of all the three isomers was done one by one in the same NMR tube so that the effect of three isomers on each other's chemical shift values can be observed. First 10 μl pinacolyl methylphosphonic acid was taken in NMR tube in D_2O and pH was maintained at neutral by adding 10% alkaline D_2O solution. It showed signal at 25.138 ppm, now 10 μl 1-methylpentylmethylphosphonic acid was added (prepared in neutral pH) in the same solution and NMR Spectrum was recorded. It showed an additional signal at 25.289 ppm. Finally, 10 μl of 4-methylpentyl methylphosphonic acid was added in the same NMR tube by maintaining neutral pH. It showed one more additional peak at 26.607 ppm along with two signals of previously added chemicals. There was no variation in their chemical shift values although they were close lying after addition of chemicals in the same NMR sample tube as addition was done at low concentration and at neutral pH. At low concentration and at neutral pH chemical shifts values were exactly matched with the reference chemicals and this is the important criteria of identifying the chemicals in the spiked tests samples by NMR. Further structures of chemicals were re-confirmed by doing the standard addition of reference sample (diluted compounds) in standard test sample (standard

addition technique). In the standard addition, the intensity of a particular signal was increased after addition of a known reference chemical in test sample, which assigned the structure of a particular compound. After confirming the structure of one of the three compounds, remaining two compounds were also confirmed by applying same technique. The δ value of spiked chemicals was matched with reference chemicals as shown in Figure 7(B) for the pinacolyl methylphosphonic acid (25.138 ppm), 1-methylpentyl methylphosphonic acid (25.289 ppm), and 4-methylpentyl methylphosphonic acid (26.607 ppm).

Water sample 2

The water sample 2 was analyzed like water sample 1. It indicated the presence of one organophosphorus compound by showing only one signal in ^{31}P NMR analysis. Corresponding blank sample did not show any signal. Second water sample (W-2) showed the presence of one signal at 30.145 ppm in ^{31}P{^{1}H} NMR analysis even after concentrating the sample (Figure 8(A)). The chemical shift value at neutral pH matched with reference compound isopropylphosphonic acid as shown in Figure 8(B). Proton coupled (^{31}P) NMR of concentrated W-2 sample showed the coupling of phosphorus with protons of alkyl group and showed complex multiplicity of eight peaks [41]. On matching the spectrum with coupled library spectrum, the structure of isopropylphosphonic acid in second water sample was confirmed. Further optimized 2D HSQC NMR technique was used for confirmation of structure of spiked chemical (Figure 9). The cross peaks at 30 ppm of phosphorus signal showed bond connectivity with methyl at 1.04 ppm and methine proton at 1.57 ppm of isopropyl group and confirmed the structure.
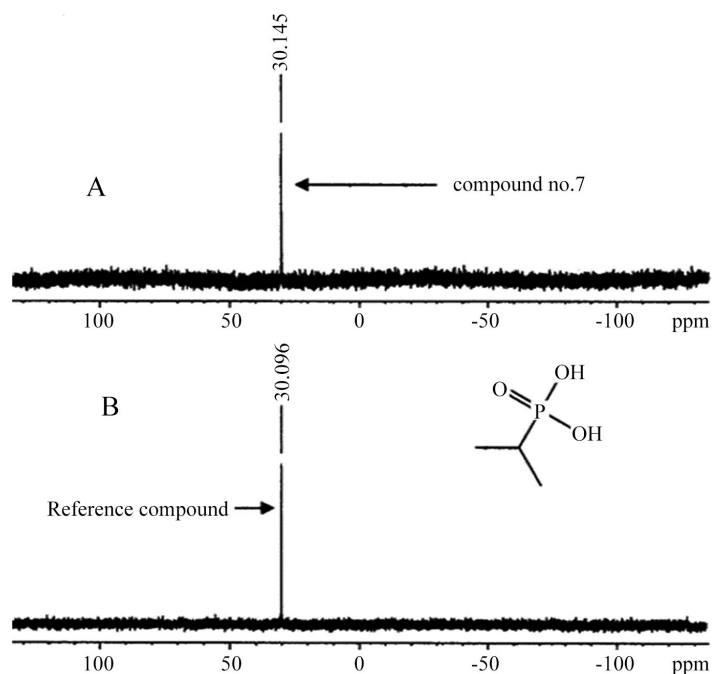

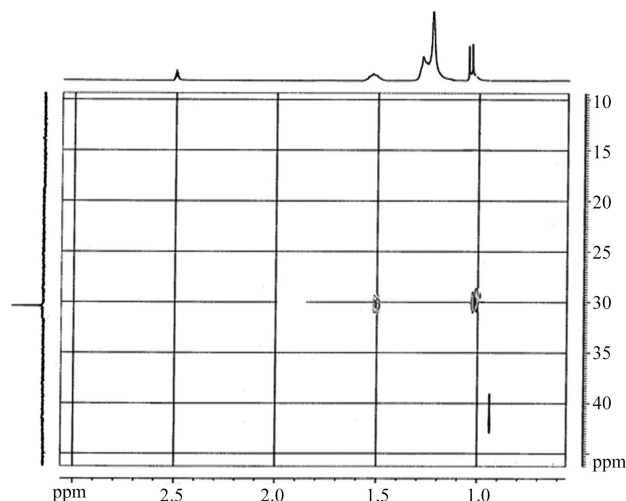**Figure 8.** ^{31}P{^{1}H} NMR Spectra of Water Sample 2 (A), Reference Chemical Isopropylphosphonic acid (B).

Figure 9. 2D ^{1}H-^{31}P{^{1}H} HSQC Spectrum of Water -2 Sample showing cross peaks for Isopropylphosphonic acid.

Result of Nineteenth Official Proficiency Test

The results obtained from OPCW [45] showed that three organophosphorus compounds 4-methylpentyl methylphosphonofluoridate, 2-isopropyl 1,3,2-dioxa-phosphophinone-2-oxide, dipinacolyl dimethyl pyrophosphonate in organic liquid, four organophosphorus compounds methylphosphonic acid, pinacolyl methylphosphonate, 1-methylpentyl methylphosphonate, 4-methylpentyl methylphosphonate in water sample (W-1) and one organophosphorus chemical isopropylphosphonic acid in water sample (W-2) were spiked. Due to the non-availability of required spectra of pyrophosphonate and phosphophinone oxide, we could not identify these two compounds and missed. This indicated that the development and strengthening of in-house library of chemicals is extremely necessary for successful performance in these tests.

As our research was focused to develop the new, effective and fast 1D and 2D NMR techniques for the structure determination of CWC related compounds at trace level in mixtures of environmental samples in the field of NMR, hence we tried to perform the post analysis of missed compounds by NMR to find out the causes of misinterpretation.

3.3. Post Test Analysis of Samples of 19th OPTs

Organic Liquid

2-Isopropyl 1,3,2-dioxaphosphophinone-2-oxide and dipinacolyldimethyl pyrophosphonate were synthesized in our laboratory and ^{31}P{^{1}H} NMR analysis was done, which showed chemical shift values at 33.60 ppm and 21.48 ppm in CDCl$_3$ respectively. Dipinacolyldimethyl pyro-phosphonate showed twelve signals of diastereomers as shown in Figure 10, (with expansion) due to four chiral centers. After characterization, we did in-house exercise by making 10 ppm solution of pyro compound. Its sensitivity and signal intensity further gone down 10 to 12 times at 10 ppm level. Hence pyro phosphonates with more than
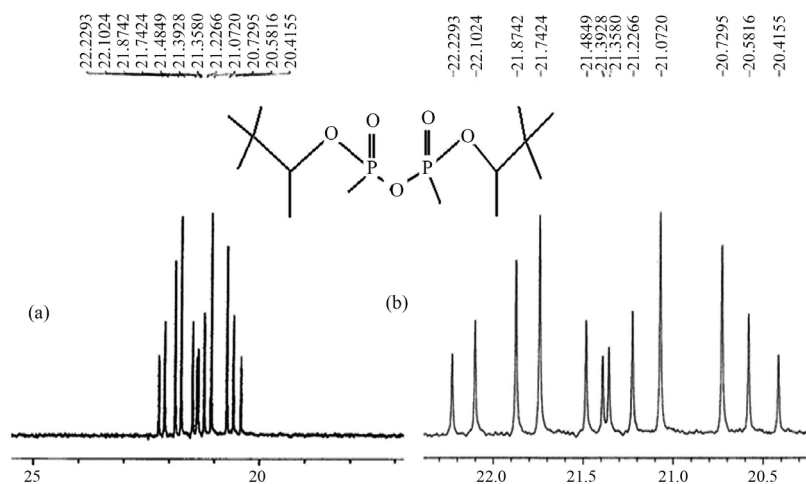
Figure 10. ³¹P{¹H} NMR Spectrum of Dipinacolyl dimethylpyrophosphonate (a), and its Expanded spectrum (b) in CDCl₃.

two stereogenic centers with multiple low intense signals create hurdle in identification in trace level analysis. This was the reason of getting multiple signals of low intensity of ³¹P{¹H}signals in the test sample. Hence it can be deduced that identification of multiple signals of Pyrophosphonates using NMR is very difficult. The chemical shift value of synthesized 2-isopropyl 1-dioxaphosphophinone-2-oxide at 33.60 ppm matched with spiked sample but it created confusion with Chemical shift value of Pinacolyl methylphosphonate at 33.2 ppm in CDCl₃. The dipinacolyldimethyl pyrophosphonate gave pinacolyl methylphosphonate after degradation in sample or it may get degraded in GC column used in GC/MS, which was actually obtained in our analysis at 29 ppm in NMR and also in GC/MS analysis. So in this organic sample we reported one chemical pinacolyl methylphosphonate wrongly and missed 2-isopropyl 1,3,2-dioxa-phospho-phinone-2-oxide, and dipinacolyldimethyl pyrophosphonate in reporting due to insufficient data. Only one compound 4-methylpentyl methylphosphonofluoridate was reported correctly in organic liquid.

Water 1 sample

Among the four signals, one broad signal at 20.88 ppm was little confusing as in this range phosphoramidates fall but in the CDCl₃ solvent. During analysis this signal was missed and not reported. Later this signal was assigned for methylphosphonic acid in OPCW result. Methylphosphonic acid showed signal at 20.19 ppm (pH 14, conc. 10 mg/ml), and 30.15 ppm (pH 1, 50 mg/ml) in D₂O and at 26.4 ppm/31.03ppm in DMSO and CDCl₃ solvent (40 mg/ml) respectively. δ (Chemical shift) at 25.12 ppm at neutral pH in D₂O and 28.29 ppm in CD₃OD is also observed. Water 1 sample at neutral pH showed signal at 20.88 ppm, while methylphosphonic acid shows signal at this ppm at pH 14. This created confusion. Series of experiments were carried out in post sample analysis to understand the trend of δ with variation in concentration and pH. Concentration was changed from 10,000 ppm to 10 ppm, likewise pH changed from acidic

to basic and phosphorus chemical shift was observed which is shown in the **Figure 12**. In this regard, a 10,000 ppm methylphosphonic acid solution was prepared at acidic pH which showed signal at δ 30.13 ppm, while at 1000 ppm showed signal at 27.411 ppm, after neutralization signal shifted to δ 25.23 ppm. However, when 1000 ppm solution was diluted ten times, *i.e.*, at 100 ppm, NMR signal observed at δ 24.331 ppm because of dilution effect. This solution was not neutralized but it showed neutral pH. Now this was further diluted ten times (10 ppm) and showed signal at δ 20.751 ppm. This shows that phosphorus acids on dilution exhibit pH near to neutral (6 - 7). This further confirms that there is a significant role of concentration, pH on chemical shift value of phosphorus acids, which is ~10 ppm. Low concentration was the reason of same chemical shift value of 20.88 ppm of MPA at neutral (low concentration) and basic pH (high concentration). The solvent and concentration effect on phosphorus chemical shift value is shown in **Figure 11** and **Figure 12** respectively.
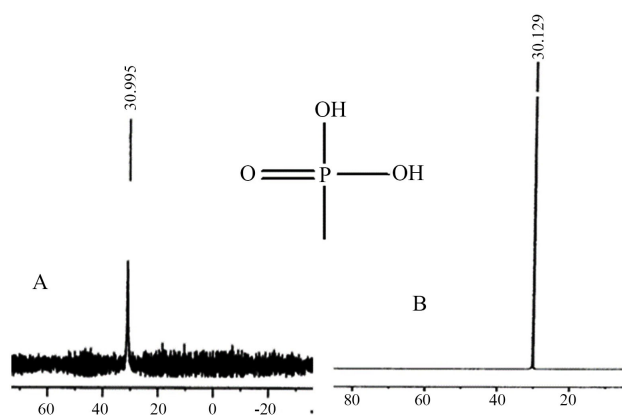

Figure 11. $^{31}P\{^{1}H\}$ NMR Spectra of Methylphosphonic acid in $CDCl_3$ (A) and in D_2O (B) showing solvent effect on chemical shift.
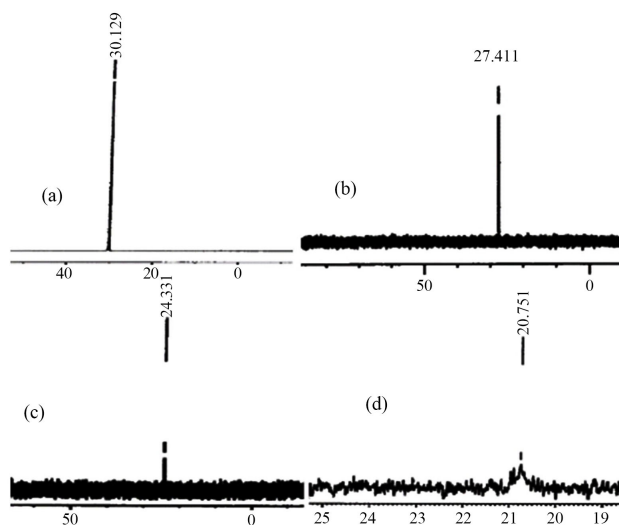

Figure 12. $^{31}P\{^{1}H\}$ Spectra of Methylphosphonic acid in D_2O at 10,000 ppm after one scan (a), 1000 ppm after one scan (b), 100 ppm after one scan (c) and at 10 ppm (d) scanned after 10,000 scans.

4. Conclusions

Development of efficient procedures for retrospective detection and identification of chemical warfare agents (CWAs) and related compounds is a prime requirement for regulation of strict verification program of Chemical Weapons Convention (CWC). The expertise gained by participation in OPTs, our lab got the status of designated laboratory by OPCW. Furthermore, this is essentially required for the country, to identity the compounds and to prove unequivocally the use, to the world body, in case, of any CW agent attack by terrorists or use of agent by adversaries in the battle field. The results are acceptable to world body only, when laboratory is accredited by a national/International body and designated by the OPCW.

During the analysis of 19th test sample it was also realized that there is difficulty in deciphering the right structure of pyro phosphonates at trace level in mixture with huge background, by NMR. Phosphorus Signal intensity of diasteromers get reduced several times due to multiple signals and low trace level，make identification further more difficult. This post analysis would help to understand the effect of chemicals, effect of pH, concentration and effect of solution mixture on NMR Chemical shift, splitting pattern and provide direction for identification of compounds in OPTs. Further this analysis would decide the road map for identification of compounds in mixtures.

Acknowledgements

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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