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Review

Use of NMR techniques for toxic organophosphorus compound profiling☆

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ABSTRACT

This review presents with selected examples the versatility of nuclear magnetic resonance (NMR) spectroscopy in the analysis of toxic organophosphorus (OP) compounds, i.e. OP pesticides and chemical warfare agents (CWAs). Several NMR applications of biological importance, like studies on inhibition mechanism, metabolism, and exposure determination, are presented. The review also concerns with the environmental analysis of OP compounds by NMR spectroscopy. Residue analysis of environment and food samples as well as characterization of degradation in environment is discussed. Some of the NMR studies that have been done to support the Chemical Weapons Convention, i.e. the development of suitable CWA detoxification means and the method development of verification analysis for CWAs and their degradation products, are outlined.

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Contents

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1. Introduction

Organophosphorus (OP) compounds are derivatives of phosphorus that have at least one organic (alkyl or aryl) group attached to the phosphorus atom either directly or indirectly by means of another element (e.g. oxygen, sulfur or nitrogen) [1]. OP compounds are in many cases highly toxic, and some of these toxic OP compounds have importance as pesticides. Pesticide is a broad term, covering a range of products that are used to control pests: insect killers (insecticides), mould and fungi killers (fungicides), weedkillers (herbicides), slug pellets (molluscicides), plant growth regulators, bird and animal repellents, and rat and mouse killers (rodenticides) [2]. National regulations control the availability of pesticides on market, and define the acceptable upper limits of the amounts of pesticide residues in food products and animal feed. A part of the toxic OP compounds has gained notorious reputation due to their potential as chemical warfare agents (CWAs) [3,4]. The CWAs have usually been discovered in connection with the development of pesticides. The G-series nerve agents, tabun (ethyl dimethylphosphoramidocyanidate, GA), sarin (isopropyl methylphosphonofluoridate, GB), soman (pinacolyl methylphosphonofluoridate, GD), and cyclosoman (cyclohexyl methylphosphonofluoridate, GF), were discovered during the 1930s and 1940s, and the V-series nerve agents like VX (*O*-ethyl *S*-(2-diisopropylaminoethyl) methylphosphonothiolate) and its isomer Russian-VX (*O*-isobutyl *S*-(2-diethylaminoethyl) methylphosphonothiolate) later during the 1950s [3,4]. The international concern over the threat of CWA culminated in 1993 to an agreement, the Chemical Weapons Convention (CWC) [5], which prohibits the development, production, acquisition, stockpiling, retention, transfer and use of chemical weapons. The Technical Secretariat of the Organisation for the Prohibition of Chemical Weapons (OPCW) [6] is the governing body that implements the CWC internationally. While general pesticides are not included as Scheduled Chemicals in the CWC [5], they can be as harmful as CWA to humans, and occasionally they have been weaponized [7,8]. However, according to Article VI of the CWC [5], States Parties must adopt measures to ensure that any toxic chemicals and their precursors are only used for purposes not prohibited by the CWC. The States Parties which have chemical plants or other facilities producing certain amount of toxic OP compounds must declare their production to the OPCW. The State Party must grant to the OPCW inspectors access to facilities as required in the Verification Annex of the CWC [5].

The introduction of pesticide residues in the environment through agricultural processes is a major public concern [9]. Abandoned CWA munitions can also pollute the environment [10]. This can result in that humans, domestic animals, as well as wildlife can be exposed to harmful doses of OP compounds. Valid analytical techniques are needed to monitor that the level of toxic OP compounds in the environment follows the regulations, and to determine the cause of poisoning when a harmful level of exposure has occurred. Separation techniques hyphenated to detectors with a high sensitivity, like gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS), have usually been the methods of choice [11]. Nuclear magnetic resonance (NMR) spectroscopy as one of the most important structural elucidation techniques has also been employed in the OP compound analyses. The strength of NMR spectroscopy has been in characterization of the chemical structures [12], and by that giving information about the OP compound degradation processes in the environment as well as the OP compound metabolism in organisms. Because NMR is also a quantitative technique [13], it has been applied in quality control of the OP pesticides and other agrochemical products [14]. Finally, NMR is nondestructive, meaning that the sample can be analyzed without consuming it during the process like with GC–MS or LC–MS techniques, and the sample can be stored after the analysis for later studies.

The NMR spectroscopy of phosphorus-containing chemicals can be considered to begin from the discovery of the nuclear resonance of phosphorus [15]. First notion about the characteristic J_{PF} coupling of phosphorus–fluoride compounds was then reported by Gutowsky and McCall [16]. Muller et al. [17] reported ^{31}P shifts of 63 different OP compounds, and the relation between the chemical shift and the structure was discussed on theoretical basis. Since then, ^{31}P NMR spectroscopy [18,19] has established its usefulness in the analysis of OP compounds.

One of the reasons for the popularity of ^{31}P NMR spectroscopy is the relatively good sensitivity of phosphorus. Phosphorus-31, a half-spin nucleus, exists on 100% natural abundance. Its receptivity is roughly 400 times higher compared to carbon-13 on 1.1% abundance. Furthermore, the chemical shift of phosphorus is very sensitive to its chemical environment, and offers a reliable way to identify the OP compounds even in complex mixtures. The chemical shift range of phosphorus is quite broad (ca. 2000 ppm) [20], and background signals do not usually obscure the relevant OP compound peaks like in the ^{1}H NMR analyses. The ^{31}P detection can be used also with solid samples using magic angle spinning (MAS) techniques [21], thus offering a way for direct analysis of soil samples.

On some occasions the amount of OP compound can be scarce, and ^{1}H NMR has been found useful due to its higher sensitivity compared to ^{31}P detection, although the background signals can hamper compound identification. There are also demonstrations how ^{1}H-^{31}P correlation spectroscopy can be used for both sensitive and selective screening of the OP compounds. Two-dimensional (2D) NMR [22], when used with a mixture, can act as a "separation" technique to distinguish different components as well as isomers in the mixture. There are also some recent examples how an established separation technique, liquid chromatography [23], can be hyphenated to NMR in OP compound-related analyses.

This review will not go into the details of the NMR experiments used, as there are many good text books about the NMR theory and techniques (e.g. [24,25]). The main focus of this review is to highlight with selected examples how NMR has been applied in the analysis of toxic OP compounds, e.g. pesticides and CWAs (Table 1). The topic has been divided on the basis of the applications. First the text will focus on the biological aspects of OP compounds, e.g. the enzyme inhibition mechanism, metabolites, biomonitoring, and antidotes, and will outline some of the NMR studies on the topics. The environmental fate of the OP compounds has been of concern for some time due to raising environmental awareness. While the application of NMR spectroscopy in environmental chemistry is well described elsewhere [26], some of the applications in detection of the OP compounds in environmental and food samples, as well as characterization of OP compound degradation in environment will be presented. The last sections are more concerned with CWAs, and will outline some of the NMR investigations that have been conducted during the development of suitable CWA detoxification means, as well as in the verification of CWAs and their degradation products in environmental samples and urban matrices.

2. Applications

2.1. Enzyme inhibition mechanism

The high toxicity of OP compounds is due to a cascade of reactions that begins with inhibition of acetylcholinesterase (AChE), a serine hydrolase responsible for processing the neurotransmitter acetylcholine. The inhibition is caused by formation of a stoichiometric (1:1) covalent conjugate with the active site serine. This

Table 1
Summary of the reviewed NMR studies.

Application	Target compounds	Information provided by NMR	NMR techniques used	References
Enzyme inhibition mechanism	Chymotrypsin	OP conjugate stereochemistry	^{31}P	[31,32,33,40]
	Chymotrypsin	OP conjugate stereochemistry, OP conjugate aging	^{31}P	[34,35]
	α-Lytic proteinase, chymotrypsin, chymotrypsinogen, trypsin, trypsinogen	Correlation of chemical shift and pH	^{1}H, ^{31}P	[36]
	Chymotrypsin, chymotrypsinogen, atropinesterase, subtilisin	OP conjugate aging	^{31}P	[37]
	Trypsin, chymotrypsin, subtilisin	Characterization of binding site	^{31}P	[38]
	AChE, BChE, chymotrypsin	Characterization of binding site, OP conjugate aging	^{31}P	[39]
	Tripeptides	Reference data	^{31}P	[41]
	Diisopropyl fluorophosphatase	Characterization of binding site	^{31}P	[42]
	Chymotrypsin	OP conjugate ageing	^{31}P, diffusion-edited ^{31}P	[44]
	Trypsin, trypsinogen	Characterization of binding site, protonation states	^{1}H	[45]
Toxicity studies	Fenitrothion	Purity of chemicals	^{31}P	[60]
	Several OP pesticides	Purity of chemicals	^{31}P	[61]
	Isoparathion methyl	Stereochemical purity of chemicals	^{31}P	[62]
	Profenofos	Stereochemical purity of chemicals	^{1}H	[63]
	Phosphorothiolates	Identification of sulfur-containing oxidation products	^{1}H, ^{13}C, ^{31}P	[64]
	Phosphonofluoridates	Purity of chemicals	^{1}H, ^{31}P	[66]
	Acephate, methamidophos	Identification of metabolites	^{31}P	[68]
	$POCl_3$	Identification of reaction products	^{31}P	[70]
Biomonitoring of human exposure	Malathion	Identification of metabolites	^{1}H	[76]
	Chlorpyrifos	Identification of metabolites	^{1}H	[77]
	Vamidothion	Identification of metabolites	^{1}H	[78]
	Phosphorodithioates	Identification of metabolites	^{31}P	[79]
	FP-biotin	Compound stability, AChE and BChE binding	^{31}P	[80]
	Chlorpyrifos	Changes in energy metabolism	^{31}P, 2D ^{31}P–^{1}H heteroTOCSY	[83]
	Glyphosate	Trace analysis of biomedical samples	^{1}H, ^{31}P	[87–90]
Studies on OP compound antidotes	Obidoxime	Identification of OP reaction products	^{31}P	[95]
	Phosphoryl oxime	Identification of OP reaction products	^{31}P	[96]
	Phosphotriesterase, paraoxonase	Purity of synthesized OP compounds	^{1}H, ^{13}C, ^{31}P	[99]
	Albumin	Identification of OP reaction products	^{31}P	[100]
	BChE	Interaction with fluoride ion	^{19}F	[101]
Pesticide residue analysis in environment and food	Several OP compounds	Identification of compound class	^{31}P	[104]
	Several OP compounds	Detection limit	^{31}P	[105]
	Several OP pesticides	Trace analysis in food	^{31}P	[106]
	Dialkyl phosphoserine peptides	Identification of compounds	^{1}H, ^{31}P	[107]
	Glyphosate	Identification of compounds	^{31}P	[109]
	Trichlorfon	Trace analysis in food	^{31}P	[110]

Table 1 (*Continued*).

Application	Target compounds	Information provided by NMR	NMR techniques used	References
Degradation in environment	Dichlorvos	Degradation monitoring	^{31}P	[121]
	Phorate	Degradation monitoring	^{31}P	[122]
	Acephate, azinphos-ethyl, demeton-S, malathion, phosalone	Degradation monitoring	^{31}P	[123]
	Sulprofos	Degradation monitoring	^{1}H, ^{13}C, ^{31}P	[124]
	Glyphosate	Degradation monitoring	^{31}P	[125,126]
	Methyl parathion, chlorpyrifos	Degradation monitoring	^{31}P MAS	[127–129]
	VX	Degradation monitoring	^{31}P MAS	[131]
Degradation in detoxification solutions	VX	Degradation monitoring	^{31}P	[139]
	Soman, VX	Degradation monitoring	^{31}P	[140]
Verification analysis	Several OP nerve agents	Reference data	^{1}H, ^{19}F, ^{31}P	[147]
	Octyl sarin, octyl methylphosphonic acid	Identification of compounds	^{19}F, ^{31}P	[149]
	Several CWC-related OP compounds	Identification of compounds	^{1}H, ^{31}P	[150,151]
	Alkyl methylphosphonic acids	Identification of compounds	^{1}H, ^{13}C, ^{31}P	[154]
	Amiton	Identification of degradation products	^{31}P	[155]
	Phosphorothioates, other OP compounds	Identification of compounds	^{1}H, ^{31}P, COSY, 2D ^{31}P-^{1}H HETCOR	[157]
	Tabun and related OP compounds, phosphonates	Identification of compounds	2D ^{1}H-^{31}P HSQC, 2D ^{1}H-^{31}P HSQC-TOCSY	[163]
	Phosphonic acids, phosphonates, other OP compounds	Identification of compounds	2D ^{1}H-^{31}P fast-HMQC, 1D ^{31}P-selective ^{1}H-^{31}P HSQC-TOCSY	[165]
	Tabun and related OP compounds, phosphonates, phosphonic acids	Screening of compounds	1D ^{1}H-^{31}P HSQC, 1D ^{1}H-^{31}P HSQMBC	[166]
	Alkyl methylphosphonic acids, methylphosphonic acid	Screening of compounds, quantification	1D ^{1}H-^{31}P HSQC	[168]
	Trimethyl phosphate, methyl ethylphosphonic acid, dimethyl ethylphosphonate	Screening/identification of compounds	LC–NMR (on-flow ^{1}H{^{31}P}), TOCSY	[171]
	Alkyl methylphosphonic acids, methylphosphonic acid	Screening/identification of compounds	LC–SPE-NMR (on-flow 1D ^{1}H-^{31}P HSQC), ^{1}H	[173]
	Derivatized phosphonic acids	Screening/identification of compounds	LC–UV–NMR (on-flow ^{1}H)	[175]

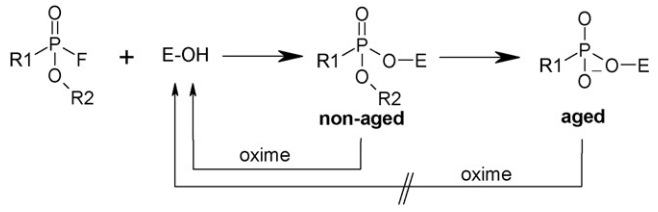

Fig. 1. Chemical pathways of the inhibition, reactivation, and aging of serine hydrolases (E–OH) in reaction with phosphonofluoridates and phosphorofluoridates [29,30].

causes excess of acetylcholine to accumulate in nerve synapses, leading to nerve overstimulation [27]. The symptoms, depending on the level of exposure, are hypersecretions, fasciculations, tremor, convulsions, coma and death [28]. In general, the inhibited cholinesterases can be reactivated by various oxime nucleophiles, but for certain OP compounds, the displacement of the phosphoryl moiety from the active site to restore the enzyme activity is impossible due to the parallel aging reaction (Fig. 1) [29,30].

^{31}P NMR spectroscopy has been indispensable in studies of the serine hydrolase inhibition induced by OP compounds. The range of application covers structural characterization of OP–chymotrypsin conjugates [31–35], pH titration studies of (diisopropylphosphoryl)serine proteinases [36], aging studies of atropinesterase and several serine proteases [37], and indirect calcium-binding site characterization of trypsin, chymotrypsin, and subtilisin [38]. Segall et al. [39] characterized the OP compound moiety of phosphorylated cholinesterases by both direct and comparative ^{31}P NMR spectroscopy. They elucidated the structure of the aged and nonaged OP conjugates of AChE and butyrylcholinesterase (BChE) that were reacted with soman, methylphosphonodifluoridate, and diisopropyl phosphorofluoridate. This, together with the characterization of a homologous pair of OP–chymotrypsin conjugates, permitted speculation on the mechanism of the reactivation and aging of OP–cholinesterases and offered a partial explanation for the resistance of aged conjugates to reactivation.

The reaction of OP compound racemate with serine hydrolases usually expresses biexponential time dependence due to the chiral selectivity of enzymes, resulting in a diastereomeric mixture of OP–enzyme conjugates. In a study by Kovach et al. [40] ^{31}P NMR spectroscopy was used to analyze chymotrypsin conjugates of racemic OP compound. According to the authors, separate ^{31}P resonances were observed for the first time by ^{31}P NMR kinetic measurements from IMN (4-nitrophenyl 2-propyl methylphosphonate) conjugate of chymotrypsin. The resonances were explained to be from the diastereomeric chymotrypsin conjugates of the two enantiomers of IMN.

Determination of an individual phosphorylation mechanism is challenging and further complicated by the difficulties associated with the analysis of high molecular weight proteins. More simplified model systems can help to reveal the inhibition and reactivation pathways. A typical approach in studies of mechanisms of cholinesterase inhibition and reactivation has been to use tripeptide sequences containing the active serine residue. Thompson et al. [41] reported ^{31}P chemical shifts of several OP compounds covalently bonded to the serine of a tripeptide Glu–Ser–Ala, which was used as a truncated form of the human serum cholinesterase active site. The authors were positive that the reported ^{31}P chemical shifts would provide useful standards for the NMR analysis of OP compounds inhibition mechanisms.

Blum et al. [42] studied phosphotriesterase mechanism of diisopropyl fluorophosphatase, a known phosphotriesterase enzyme from *Loligo vulgaris*. In this multidisciplinary study they applied ^{31}P NMR to characterize dynamics of the *O,O*-dicyclopentylphosphoroamidate binding to the active site (Fig. 2).

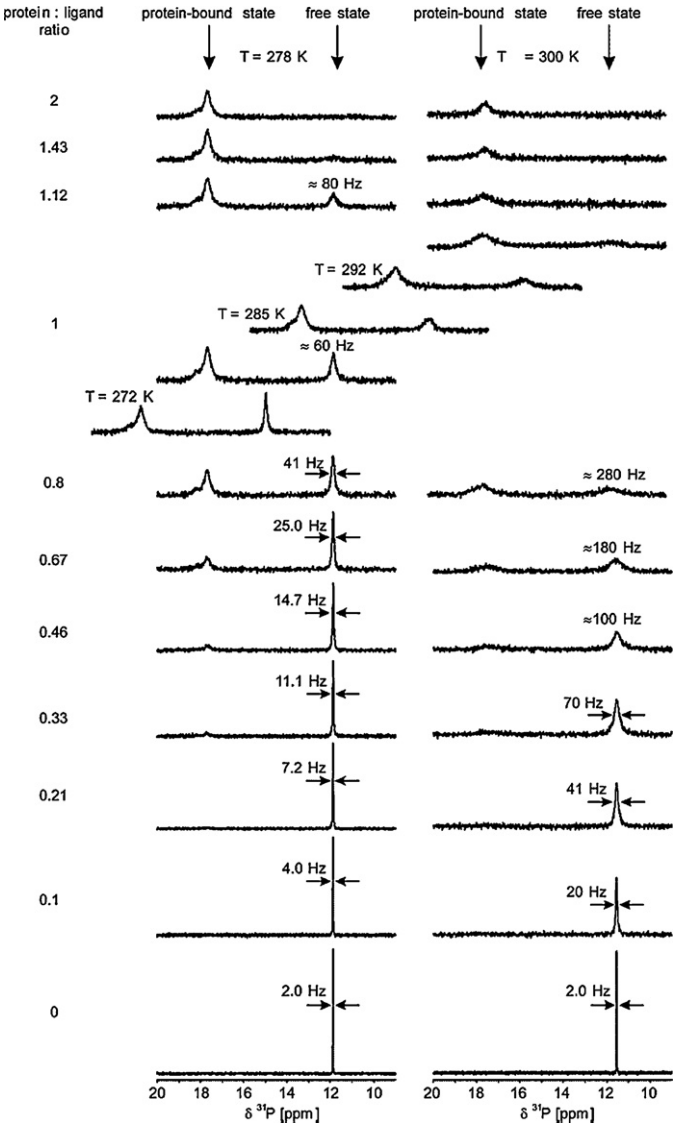

Fig. 2. 242 MHz ^{31}P{^{1}H} NMR spectra from titration of *O,O*-dicyclopentylphosphoroamidate with diisopropyl fluorophosphatase at 278 and 300 K. The protein–ligand ratio is indicated on the left. For a 1:1 mixture, spectra were recorded at five different temperatures. A second signal at 5.8 ppm downfield from the free OP compound that becomes visible upon addition of the enzyme is from the OP conjugate. The appearance of two separated signals was explained to be due to a slow exchange of the free and bound states on the NMR time scale, with an estimated upper limit of 1400 s^{-1}. The change of the chemical shift was explained by the polarization of the phosphorus–oxygen bond due to coordination to the calcium, indicating that the calcium ion participates in reactivation.
Reprinted with permission from [42]. Copyright 2006 American Chemical Society.

Based on the computational and experimental results they proposed an alternative reaction mechanism from the previously reported one [43]. The proposed reaction mechanism involved calcium coordinating residue D229 as a nucleophile and a phosphoenzyme intermediate.

An interesting aspect of the study by Segev et al. [44] was the application of the diffusion-edited ^{31}P NMR spectroscopy to resolve OP compounds that where either in free state, in OP–chymotrypsin conjugate, or in aged OP–chymotrypsin form. They demonstrated that the diffusion NMR spectroscopy offers an efficient tool for the screening of protein–ligand reversible interactions without the need for ligand removal or specific conjugate isolation.

While the major part of the studies of enzyme inhibition mechanism has monitored the OP compound in the inhibition process, NMR spectroscopy has also been used to monitor the enzyme and its structural changes in the OP inhibition. Porubcan et al. [45] applied ^{1}H NMR in pH titration studies of porcine and bovine trypsins and trypsinogens. They monitored the active site histidine-57 proton resonances of trypsins and trypsinogens inhibited either with diisopropyl phosphorofluoridate or bovine pancreatic trypsin inhibitor in order to define the protonation states of the active site amino acids in zymogens. Steitz and Shulman [46] reviewed crystallographic and NMR spectroscopy studies on the structure and enzymatic mechanism of serine proteases, and presented some ^{1}H NMR, ^{13}C NMR, and ^{15}N NMR data. These nuclei are utilized in the general NMR methodology for the structural characterization of biomacromolecules, and the research field has developed a great deal in last decades. Special experimental techniques like transverse relaxation optimized spectroscopy (TROSY) [47] and cross-correlated relaxation-enhanced polarization transfer (CRINEPT) [48,49], protein site-specific labeling [50], and use of residual dipolar couplings as structural constrains [51] have further pushed the envelope of the molecular size limit, and an NMR structure of protein with molecular weight exceeding 80 kDa has been reported [52]. Nowadays the structure determination of proteins is one of the most important applications of NMR spectroscopy [53,54].

2.2. Toxicity studies

The toxicity of OP compound depends greatly on the molecular structure as well as the stereochemistry. OP compounds can also have synergistic properties; it is known that the presence of other OP compounds increase the toxicity of certain pesticides like malathion (*S*-1,2-bis(ethoxycarbonyl)ethyl *O*,*O*-dimethyl phosphorodithioate) to warm-blooded animals and resistant insects [55–58]. The chirality of the chemical produced in industrial processes may not be well defined, and the technical grade pesticides from formulation or after improper storage [59] can also contain unknown amounts of impurities. These deviations in the pesticide product can lead to unintended effects in the use of pesticides. NMR spectroscopy as a powerful structural elucidation technique has been in an important role in characterization of pesticide stereochemistry and purity [60,61], and by that, explaining the changes in the toxicity of pesticides.

Ryu et al. [62] studied the importance of stereochemistry in the relative inhibition potency of the chiral isomers of isoparathion methyl (*O*-(4-nitrophenyl) *O*-methyl *S*-methyl phosphorothioate) with four different cholinesterases. They applied ^{31}P NMR to confirm the purity of the synthesized OP compounds prior to toxicity studies. The authors found that (−)-stereoisomers had a 6–15-fold difference in inhibitory action against the cholinesterases.

John Casida and his research group have been productive contributors in the OP compound toxicity studies. Leader and Casida [63] studied biological activity of the chiral isomers of profenofos (*O*-(4-bromo-2-chlorophenyl) *O*-ethyl *S*-propyl phosphorothioate) insecticide. After synthesis of the chiral isomers of profenofos, ^{1}H NMR, with addition of a chiral shift reagent, was used in the determination of the optical purity of the compounds (Fig. 3). The chiral isomer (+)-profenofos was found to have a 23-fold higher toxicity in mice and a 4–12-fold higher toxicity in insects.

Wu et al. studied [64] the conditions to obtain sulfur-containing oxidation products of certain phosphorothiolates. The authors' interest for the corresponding sulfoxide and sulfone products was due to their potential toxicological relevance as bioactivation products of phosphorothiolate pesticides [65]. The authors reported ^{1}H, ^{13}C, and ^{31}P assignments of several intermediate products as well as the assignments of the final sulfoxide/sulfone compounds found after *m*-chloroperoxybenzoic acid treatment. In a subsequent study, Wu and Casida [66] represented a toxicity study of ethyl octylphosphonofluoridate and its analogues in inhibition of neuropathy target esterase [67]. ^{1}H and ^{31}P NMR data of the OP compounds was reported.

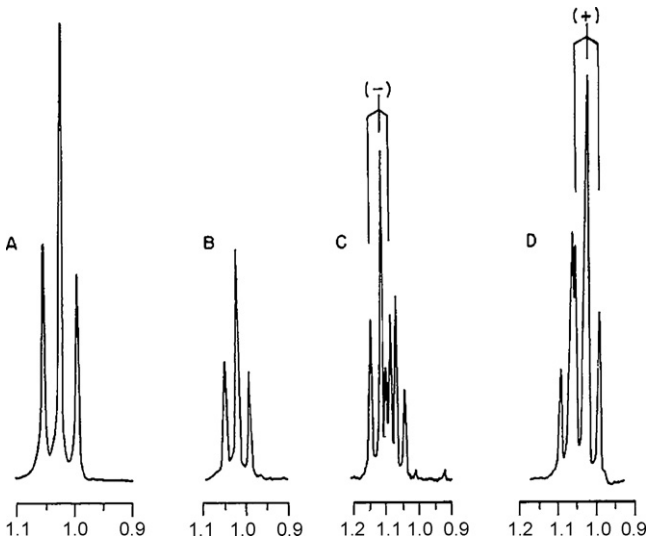

Fig. 3. 250 MHz ^{1}H NMR spectra of methyl resonances of $-SCH_2CH_2CH_3$ side chain of profenofos with shift reagent. (A) (−)-profenofos, $[\alpha]_D - 15.3^\circ$, (B) (+)-profenofos, $[\alpha]_D + 15.6^\circ$, (C) (±)-profenofos from (−)-profenofos after double inversion caused by HCl-catalyzed solvolysis, $[\alpha]_D + 5.8^\circ$, (D) (±)-profenofos from (+)-profenofos after double inversion caused by HCl-catalyzed solvolysis, $[\alpha]_D + 11.9^\circ$.
Reprinted with permission from [63]. Copyright 1982 American Chemical Society).

Mahajna et al. [68] investigated the toxicity of acephate (*O*,*S*-dimethyl acetylphosphoramidothioate). Based on the earlier studies [69], the selective toxicity in insects is attributed to a rapid biotransformation of acephate by carboxyamidase to methamidophos (*O*,*S*-dimethyl phosphoramidothioate) which acts as an AChE inhibitor, while the same biotransformation is much slower in mammals. They applied ^{31}P NMR in analysis of urine samples of rodents administered with intraperitoneal injections of acephate with or without methamidophos pretreatment. Quantification of the urinary metabolites revealed that methamidophos pretreatment greatly alters the balance of acephate metabolites in urine by doubling the total amount of the combined demethylation products from acephate. Based on the data acquired with NMR, GC–MS, and HPLC–UV, the authors proposed a hypothesis that the safety of acephate in mammals is due to its conversion in small part to methamidophos which, acting directly or as a metabolite, is a potent carboxyamidase inhibitor, thereby blocking further activation.

Quistad et al. [70] studied the toxicity of phosphorus oxychloride ($POCl_3$), an intermediate in the synthesis of many OP insecticides and chemical warfare nerve gases. They observed that hydrolytically unstable $POCl_3$ gives poisoning signs in mice after intraperitoneal injection and in fumigant-exposed houseflies similar to those produced by the OP insecticides and CWA. With aid of ^{31}P NMR spectroscopy they concluded, that the actual phosphorylating agent, phosphorodichloridic acid ($HOP(O)Cl_2$), is formed within seconds from $POCl_3$ in water. They also proposed a mechanism for electric eel acetylcholinesterase inhibition by $HOP(O)Cl_2$.

2.3. Biomonitoring of human exposure

Accidental pesticide exposure in humans happens usually through dermal exposure, oral ingestion, or inhalation, and is observed most commonly among agricultural workers [71,72].

Intentional pesticide exposure cases are generally due to the result of an attempted suicide, and are typically oral ingestions, but some exceptional cases of parenteral self-poisoning [73] have been reported. The cases of CWA exposures are rare, but some reports about the Tokyo subway attack in 1995 have been presented [74].

The OP compound metabolites that result from biotransformation in organisms [75] are used as marker chemicals in determination of the OP compound exposure. NMR spectroscopy has been an important tool in structural characterization of unknown metabolites. The metabolite structure can clarify the biotransformation pathways that the toxic OP compound undergoes in organisms. The metabolism studies are usually carried out *in vitro* in order to keep the system as controlled as possible, and the concentration of OP compounds on the level that facilitates NMR analyses. The concentration level of metabolites in lethal poisoning cases can also be sufficient for NMR studies.

Chen et al. [76] investigated carboxylesterase hydrolysis of malathion. They used IR and ^{1}H NMR to analyze the structure of the hydrolysis product, which was suggested to result from hydrolysis of one of the two carbethoxy groups of the succinate portion of the malathion molecule. The results indicated that only one of the two possible isomers, *O*,*O*-dimethyl-*S*-(1-carboxy-2-carbethoxy)ethyl phosphorodithioate, is produced.

Lores et al. [77] studied metabolism of chlorpyrifos (*O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate) in a lethal poisoning case. In course of the analysis they found a previously unreported hepatic metabolite of chlorpyrifos. The metabolite was characterized with various instrumental methods such as gas chromatography and mass spectrometry, and the structure of the metabolite was finally studied with ^{1}H NMR. The metabolite resulted in from substitution of a chlorine in the pyridinol ring with a methylthio (–SCH_3) group.

El-Oshar et al. [78] investigated the *in vitro* metabolism of vamidothion (*O*,*O*-dimethyl *S*-2-(1-methylcarbamoylethylthio)ethyl phosphorothioate) and its thio-analogue thiovamidothion in rat and mouse liver subcellular fractions. They applied several analytical techniques in the study of the major routes of metabolism. They were able to confirm with NMR spectroscopy and IR spectroscopy that vamidothion is rapidly oxidized to the sulfoxide, which was the main metabolite. Thiovamidothion was extensively oxidized to thiovamidothion sulfoxide, vamidothion, and vamidothion sulfoxide in addition to oxidative hydrolytic products. The authors demonstrated with the cytochrome P-450 inhibitors that the cytochrome P-450-dependent monooxygenase system is closely involved with the main metabolism of vamidothion and thiovamidothion.

Urinary metabolites are readily accessible biomarkers, although their rapid elimination limits their use for retrospective detection. Mahajna et al. [79] analyzed metabolism of *O*,*O*-dialkyl phosphorodithioate insecticides in mammals. Their study concerned with the possibility of *S*-methylation of phosphorodithioic acids such as $(MeO)_2P(S)SH$, resulting in *O*,*O*,*S*-trimethyl compounds as metabolites. These metabolites were thought to originate from the *O*,*O*,*S*-trimethyl impurities from the manufacturing processes. The authors applied ^{31}P NMR spectroscopy to identify the urinary metabolites in mice after the intraperitoneal administration of dimethoate (Fig. 4). The results confirmed *S*-methylation pathway for dialkyl phosphorodithioate insecticides.

Macromolecule conjugates of OP compounds found in blood and tissue samples are important biological markers of toxic OP compound exposure. Schopfer et al. [80] analyzed the suitability of FP-biotin (10-(fluoroethoxyphosphinyl)-*N*-(biotinamidopentyl) decanamide) for the search of biological markers of OP compound exposure. The biotin tag has been reported to facilitate isolation and visualization of enzymes susceptible to phosphorylation [81]. ^{31}P NMR was applied in stability studies of the FP-biotin in various solutions, confirmation of covalent binding of FP-biotin with BChE, and in analysis of stoichiometry of the FP-biotin reaction. The authors stated that FP-biotin is a mixture of two stereoisomers with approximate ratio of 50:50, and the fraction of FP-biotin that failed to react with BChE also failed to label proteins in mouse brain, suggesting that only one stereoisomer of FP-biotin is reactive.

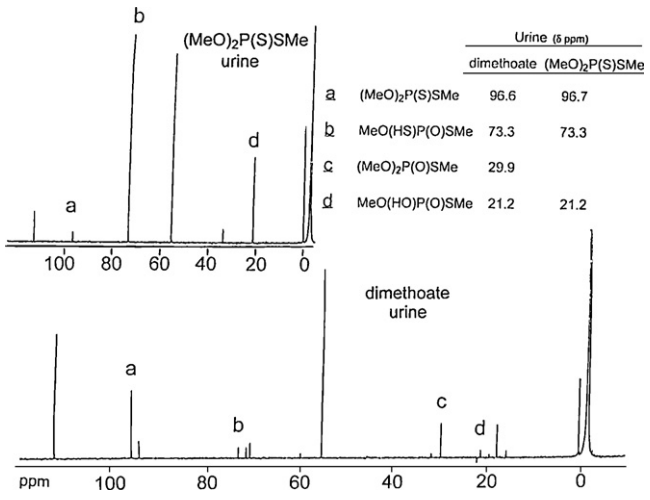

Fig. 4. 121 MHz ^{31}P NMR spectra from urinary metabolites of $(MeO)_2P(S)SMe$ and dimethoate in mice.
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The effects of OP compound exposure on organisms can also be studied indirectly. Metabonomics [82] is an emerging field of research, which is concerned with how the metabolic profile of a complex biological system changes in response to the stress like disease, toxic exposure, or dietary change. 2D NMR spectroscopy with higher resolution of the resonances and therefore better information about the sample constituent are becoming common in NMR metabonomics. Cradwell et al. [83] combined the 2D ^{31}P–^{1}H correlation spectroscopy and total correlation spectroscopy (TOCSY) [84] to identify a number of phosphorylated metabolites present in a trichloracetic acid extract of a crayfish hepatopancreas. The 2D ^{31}P–^{1}H heteroTOCSY experiment, which has also been found to be useful in assignments of nucleic acids [85], has several advantages, e.g. a single mixing period, a good sensitivity from proton detection, in-phase cross-peaks, and a phase-sensitive spectrum (Fig. 5). ^{31}P NMR spectroscopy was applied in quantification of the metabolites involved in energy metabolism (e.g. adenosine triphosphate and adenosine diphosphate) and phospholipid metabolism with respect to chlorpyrifos treatment of the crayfish. The authors, however, concluded, that none of the responses found in the changes of metabolite levels would be directly related to the known toxic mechanism of chlorpyrifos, i.e., inhibition of acetylcholinesterase, which underlines the need for examining the sublethal stress mechanism of OP insecticides in nontarget indigenous organisms.

While NMR spectroscopy has been a useful tool in characterizing the OP compound metabolite structures, application of NMR has been quite rare in direct biomonitoring of the exposure to toxic OP compounds [86]. Still, some research with NMR spectroscopy has been made in this field. Dickson et al. [87] used ^{31}P NMR in the analysis of biomedical samples from two postmortem specimens died from toxic OP compound poisoning. They were able to detect and quantify glyphosate (*N*-(phosphonomethyl)glycine) from blood, urine, and liver samples. Lhermitte and co-workers have also studied the acute intentional glyphosate poisoning using ^{1}H NMR [88,89] and ^{31}P NMR [90]. They presented how rapid and reliable detection of OP compounds in urine, serum and gastric flu-

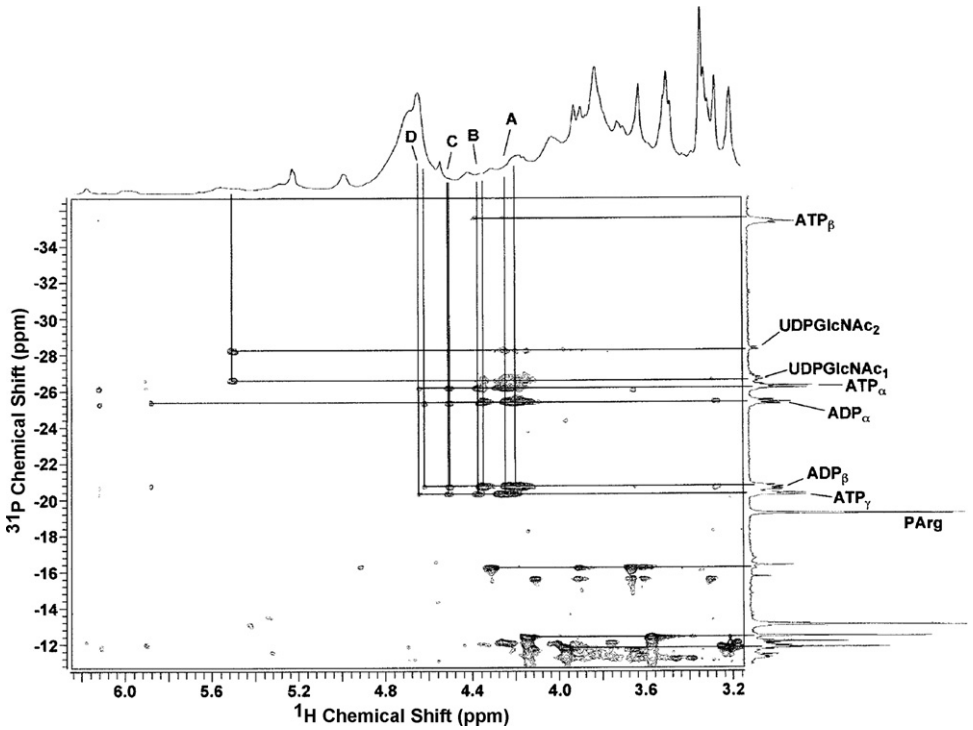

Fig. 5. Expansion of 400 MHz 2D ^{31}P–^{1}H heteroTOCSY spectrum from crayfish hepatopancreas extract. The assigned metabolites are: ATP, adenosine triphosphate; ADP, adenosine diphosphate; UDPGlcNAc, uridine-5′-diphospho-*N*-acetylglucosamine; PArg, phosphoarginine.
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ids is attainable with NMR spectroscopy [90] (Fig. 6). Furthermore, quantification of the glyphosate was possible against reference chemicals in coaxial capillary tubes. These studies demonstrate that the requirements of the sample pretreatment in NMR are much less strict than with GC–MS or LC–MS. Consequently, NMR can be a potential identification technique for the direct, non-destructive analysis of biomedical samples in acute poisoning cases, when the level of the OP compounds is high by default. However, detection of the OP compound metabolites and macromolecule conjugates in trace level exposures requires high sensitivity from the applied instrumental technique. Therefore, gas or liquid chromatography combined with tandem mass spectrometry has usually been the method of choice for identification of CWA metabolites at trace levels [91–93]. Further studies are needed to fully explore the limits of NMR spectroscopy in biomonitoring the exposure to toxic OP compounds.

2.4. Studies on OP compound antidotes

Ever since the toxic OP compounds have been weaponized, medical experts both in military and civilian healthcare have been interested in developing suitable antidotes. Current post-exposure antidotes against OP compound poisoning consist of anticholinergic drugs such as atropine sulfate, and oximes such as pralidoxime chloride (2-PAM chloride) [94]. Waser et al. [95] analyzed direct interaction of sarin with obidoxime (Toxogonin®), a drug compound which is used to reactivate partially inhibited AChE. It was reported that based on the ^{31}P NMR results obidoxime and sarin formed in a fast reaction two new compounds, single- and double-phosphorylated obidoxime. The authors concluded that obidoxime may have a detoxifying action under physiological conditions, as the phosphorylated obidoximes are less liposoluble compounds than native sarin and their distribution in the brain tissue and fat would therefore be restricted. Luo et al. [96] inspected the role of phosphoryl oxime in oxime-induced reactivation of inhibited cholinesterases. Phosphoryl oxime, an unstable intermediate, is formed during the oxime-reactivation of inhibited enzymes. This phosphoryl oxime in turn acts as a cholinesterase inhibitor, partly explaining occasional retardation of the oxime-induced reactivation of inhibited cholinesterases. The authors attempted to establish a relationship between edrophonium-induced accel-

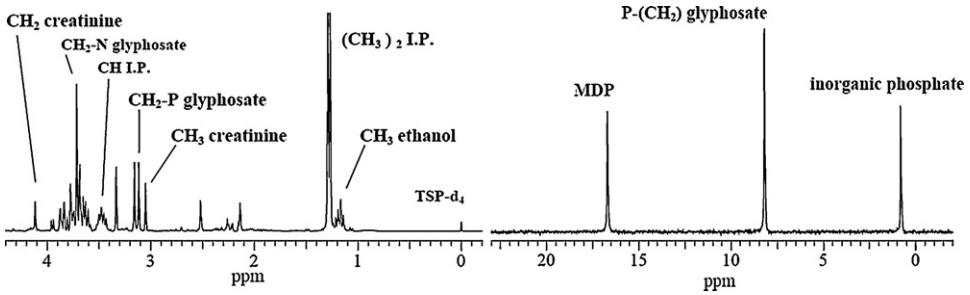

Fig. 6. Expansions of 300 MHz ^{1}H NMR (on the left) and 121 MHz $^{31}P\{^{1}H\}$ NMR (on the right) spectra from urine of a patient suffering from accidental ingestion of glyphosate. The abbreviated assignments are: I.P., isopropylamine; TSP-d4, 3-trimethylsilyl 2,2′,3,3′-tetradeuteroproprionic acid; MDP, methylene diphosphonic acid (in a coaxial capillary tube). The last two of these compounds were used as references in quantification.
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eration [97] of reactivation and phosphoryl oxime inhibition of the reactivated enzyme using several inhibited cholinesterases. ^{31}P NMR spectroscopy was used to characterize two phosphoryl oximes formed during the reactivation studies. The authors concluded that edrophonium acceleration of oxime-induced reactivation of AChE is caused by the prevention of phosphoryl oxime inhibition of the reactivated enzyme.

Recent research has focused to develop a pretreatment against OP compounds in order to suppress the toxic effects caused by the OP compound exposure. One of the approaches is to employ so-called bioscavengers to sequester highly toxic OP compounds in the bloodstream before they reach their physiological targets [98,99]. Li et al. [100] studied the potential of albumin as a bioscavenger to be used against poisoning by OP nerve agents. They applied ^{31}P NMR spectroscopy in determination of reaction kinetics and enantioselectivity of albumin inhibition (phosphorylation) by soman. They were able to determine that there was no enantiomeric preference of albumin for the soman isomers. Unfortunately, albumin reactivity with soman (phosphorylation and phosphotriesterase activity) was reported to be too slow to play a major role in detoxification.

Ashani et al. [101] studied the role of fluoride ion in BChE inhibition by OP compounds. Fluoride ion is a reversible inhibitor of BChE, and can alter the bioscavenging efficiency of BChE for OP compounds. They applied ^{19}F NMR to monitor the availability of free fluoride ions in various saline solutions and human plasma. In buffer solutions, fluoride was found to bind to BChE, thus slowing the rate of inhibition of the enzyme by OP compounds through inactivation of the enzyme. However, based in the changes in chemical shift and linewidth of fluoride resonance they concluded that there is an elevated interaction of plasma constituents with fluoride, and by that, decreased availability of fluoride to BChE. The authors concluded that BChE inhibition by OP compounds is not compromised by the normal concentration range of circulating fluoride ions, which is promising concerning with the use of BChE as a bioscavenger.

2.5. Pesticide residue analysis in environment and food

The exposure on residual amounts of pesticides currently used on farms and households may pose a certain risk, and some guidelines have recently been published about different approaches for the risk assessment [102]. The health risks from cumulative exposure are still under debate [103], and further research is needed to fully understand the implications. Due to simple requirements for the sample pretreatment, ^{31}P NMR spectroscopy has been a useful technique in monitoring the level of OP pesticide residues in the environment and food. Ross and Biros [104] demonstrated how the phosphorus chemical shift can be used to interpret the nature of the atoms bonded to phosphorus, and by that categorize pesticides to compound classes. One of the earliest references to the application of ^{31}P NMR spectroscopy in trace analysis of OP pesticides was published by Gurley and Ritchey [105]. They reported a very thorough study how a selection of OP compounds was detected at the lower parts-per-million level in aqueous test samples. With use of a suitable relaxation reagent they reduced the phosphorus T_1 times in order to improve attainable S/N within the used 25 min total acquisition time. The authors reported that a minimum detectable concentration was less than 50 μg (phosphorus) in a 2 ml sample volume (12 mm sample tube), which is quite an impressive detection limit for a contemporary 2.34 T NMR spectrometer (Table 2).

Mortimer and Dawson [106] presented a comprehensive study about ^{31}P NMR spectroscopy in the analysis of trace levels (0.1–10 parts-per-million) of OP pesticides in cole crops. A total of 24 different pesticides were analyzed with three different NMR spectrometers in the validation studies. They found a good linearity in quantification for malathion, parathion (*O*,*O*-diethyl *O*-4-nitrophenyl phosphorothioate), phosalone (*S*-6-chloro-2,3-dihydro-2-oxo-1,3-benzoxazol-3-ylmethyl *O*,*O*-diethyl phosphorodithioate), and phosmet (*O*,*O*-dimethyl *S*-phthalimidomethyl phosphorodithioate) (R^2 values are 0.9996, 0.9969, 0.9927, and 0.9865, respectively). The minimum detectable levels for malathion in a 0.4 ml sample with $Cr(AcAc)_3$ and 30-min acquisition time were 115, 27.4, and 14.4 μg/ml using 5.85, 9.36, and 11.7 T NMR spectrometers, respectively (Table 2). The test scenario with vegetable extracts demonstrated that the presence of pesticides can be revealed at 0.5 parts-per-million level from a 100 g vegetable sample, if the yield of the pesticides in organic extraction is assumed perfect (Fig. 7).

Table 2
^{1}H and ^{31}P Larmor frequencies in different magnetic field strengths.

Magnetic field strength [*T*]	^{1}H Larmor frequency [MHz]	^{31}P Larmor frequency [MHz]
2.34	100	40
4.70	200	81
5.85	250	101
7.04	300	121
9.36	400	162
11.74	500	202
14.10	600	242

Paquet and Khan [107] investigated the efficiency of supercritical fluid extraction (SFE) in plant component analysis in order to find a way to extract metabolites of OP pesticides covalently bound to serine residues of proteins in plants or grains. The phosphorylation of proteins with pesticides can reduce the nutritive value of cereal grain proteins [108]. They used model dialkyl phosphoserine peptides bound to solid support resin. The resin was subjected to the SFE process, and the released material was examined with ^{31}P NMR and ^{1}H NMR spectroscopy for the identification of dialkyl phosphate residues. The authors reported that ca. 33% of the residues were cleaved from the resin in SFE, and concluded

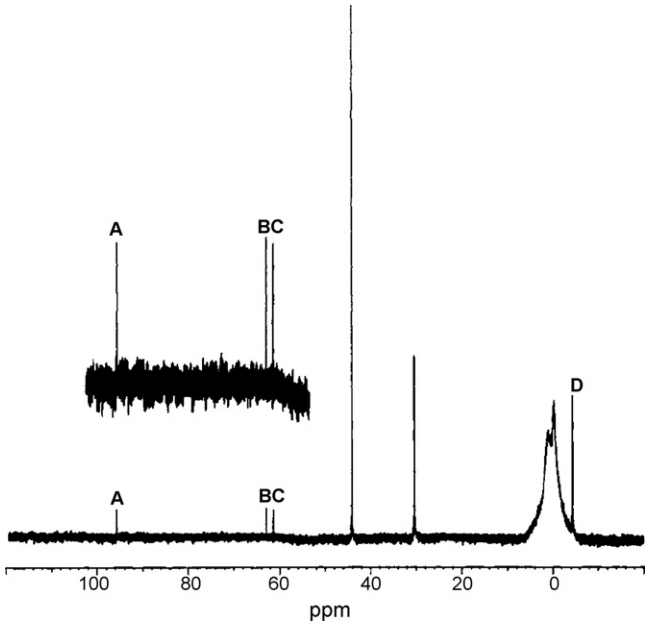

Fig. 7. 162 MHz ^{31}P{^{1}H} NMR spectrum from the concentrated red cabbage methylene dichloride extract spiked with 50 μg of disulfoton (*O*,*O*-diethyl *S*-2-ethylthioethyl phosphorodithioate) (A), parathion (B), and diazinon (*O*,*O*-diethyl *O*-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate) (C). Triphenylphosphine (D) was the internal reference standard. The sample contained also triphenylphosphine sulfide and oxide giving two additional signals at 30 and 44 ppm, and inorganic phosphates giving the broad hump at 0 ppm.
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that the combination of SFE and NMR techniques may be useful in detection of phosphorylation of biopolymers.

Kudzin et al. [109] presented a study on the detection of glyphosate and its metabolites in river water. While the authors' main interest was in the development of a GC–MS-based technique for the detection of the derivatized analytes, they also applied ^{31}P NMR spectroscopy for the preliminary analysis of the samples. Talebpour et al. [110] validated ^{31}P NMR in trace analysis of trichlorfon (*O*,*O*-dimethyl-(2,2,2-trichloro-1-hydroxyethyl) phosphonate) and its degradation products from tomato samples. The authors reported that the limit of detection was 55 mg/l without any sample pretreatment, and the linear working range for quantification was 150–5500 mg/l.

There are also other good reviews concerning with the pesticide residue analysis by NMR. A review of NMR spectroscopy in the analysis of agrochemicals and pesticides has been reported by Bright and Ratcliffe [111]. Cade-Menun [112] presented a review about the application of liquid-state and solid-state ^{31}P NMR spectroscopy in analysis of environmental and agricultural samples. The author focuses on the papers where the naturally occurring phosphorus species are studied in environment, but it is still a good reference when one needs to find details about the general applicability of NMR spectroscopy in the analysis of OP compounds. An excellent review from Spyros and Dais [113] presents how ^{31}P NMR has been applied in food analysis. The review describes the naturally existing phosphorus compounds in food, and gives some details about the ^{31}P NMR experimental conditions and sample preparation. References are given to the ^{31}P NMR analysis of vegetable oils, meat, fish, milk, plants, and some examples are presented also in food additive and pesticide residue analyses.

2.6. Degradation in environment

Halogenated organic pesticides, highly persistent chemicals [114], have caused a major impact on wildlife due to their bioaccumulation [115]. OP compounds degrade more easily in the environment [116], but the environmental impact of their degradation products is still not well defined. There are a large variety of the possible OP compound degradation pathways, of which hydrolysis and oxidation are the most typical [116], but other modes of degradation of photochemistry [117,118] and surface chemistry [119] have also been investigated. The environmental decomposition of CWA has also been under study. A good review by Kingery and Allen [120] concerns with the environmental fate of three CWA, sarin, soman, and VX. The review elaborates the different degradation pathways like hydrolysis, catalyzed reactions in solution and on solid phases, volatilization, photolysis, biodegradation, and reactions in soils, and gives a good overview the species of OP compounds that can be found in environment after CWA contamination.

NMR spectroscopy as a quantitative technique has been an important tool in the kinetic studies of OP compound degradation. Benoit-Marquié et al. [121] applied ^{31}P NMR in decomposition analysis of dichlorvos (2,2-dichloroethenyl dimethyl phosphate). They simulated environmental conditions by studying degradation kinetics and pathways in hydrolysis and TiO_2 photocatalysis. The authors reported that the hydrolysis of dichlorvos led to the formation of dimethyl phosphate (Fig. 8), while TiO_2 photocatalysis decomposed dichlorvos into dimethyl phosphate, which further reacted to monomethyl phosphate and phosphate. Compared with chromatographic methods, ^{31}P NMR spectroscopy enabled a direct study of the aqueous medium, thus avoiding the problems encountered in extraction recovery and chemical derivatization. All the phosphorylated degradation compounds were simultaneously detected and quantified in a single analysis.

Hong et al. [122] studied the kinetics profile of phorate (*O*,*O*-diethyl-*S*-ethylthiomethyl phosphorodithioate) hydrolysis with

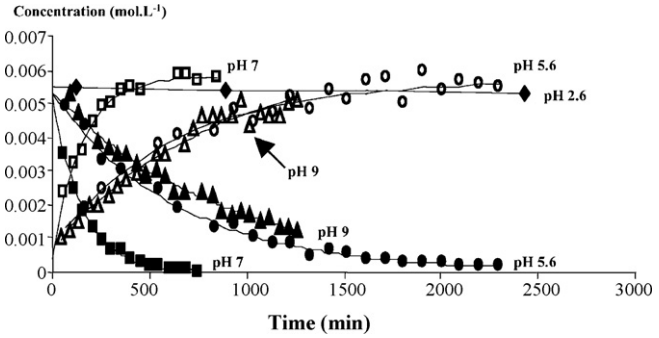

Fig. 8. 162 MHz ^{31}P{^{1}H} NMR data from the kinetic study of dichlorvos hydrolysis under different pH. Filled symbols and open symbols represent the dichlorvos and the dimethyl phosphate concentrations, respectively.
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^{31}P NMR. They observed a disappearance of the pesticide peak in accordance with pseudo-first-order kinetics. The OP compound hydrolysis product shown in the ^{31}P NMR spectrum was identified via methylation and GC–MS to be diethyl dithiophosphate. They concluded that P–S bond was left intact during the hydrolysis under the used experimental conditions, and compared their finding to the ^{31}P NMR results by Lai et al. [123], who reported that catalytic hydrolysis of five OP pesticides by organophosphorus hydrolase resulted in the cleavage of the P–S bond. Based on the ^{31}P NMR and GC–MS results, Hong et al. proposed a pathway for phorate hydrolysis under simulated natural water conditions.

OP compounds can also undergo enzymatic degradation in the environment. The application of NMR spectroscopy in the analysis of pesticide metabolism in soil was reported by Krolski et al. [124]. They studied the metabolic degradation of ^{13}C-labeled sulprofos (*O*-ethyl *O*-4-(methylthio)phenyl *S*-propyl phosphorodithioate) in biologically active sandy loam. They found that while ^{1}H NMR and ^{13}C NMR showed insufficient difference to allow the differentiation and quantification of the various compounds, ^{31}P NMR was found to be rapid, easy and accurate in the characterization and quantification of pesticide metabolites in soil (Fig. 9).

Lipok et al. [125] reported an interesting trait of cyanobacteria. Using ^{31}P NMR, they studied the ability of *Spirulina* species to degrade glyphosate. The authors reported that the cyanobacteria were able to degrade the OP herbicide glyphosate, when it was the sole source of nitrogen and phosphorus in the medium. The authors also hypothesized, that because additional peaks present in the ^{31}P NMR spectra did not correspond to the most common intermediates in glyphosate metabolization, *Spirulina* might degrade glyphosate through a pathway different from those previously elucidated in bacteria. In a latter report by Lipok et al. [126] ^{31}P NMR spectroscopy was applied in glyphosate biodegradation studies of whole cell systems *in vivo*, demonstrating the potential of NMR spectroscopy as an analytical tool in environmental biotechnology of organophosphonate xenobiotics.

Seger and Maciel have presented a number of studies [127–129] about the degradation pathways of OP pesticides sorbed on soil and clay. The interesting aspect in these studies from the point of NMR was the application of solid-state ^{31}P MAS NMR in the direct analysis of soil and clay samples. The authors applied normal cross-polarization (CP) and direct polarization (DC) techniques [130] in the studies in order to monitor mobile species or moieties (^{31}P DC/MAS) and immobile species or moieties (^{31}P CP/MAS). The examination of the samples extended over a period of several years, and the authors were able to present the predominant as well as the secondary modes of degradation of methyl parathion (*O*,*O*-dimethyl *O*-4-nitrophenyl phosphorothioate) and chlorpyrifos. A related application of ^{31}P MAS NMR in CWA degradation studies

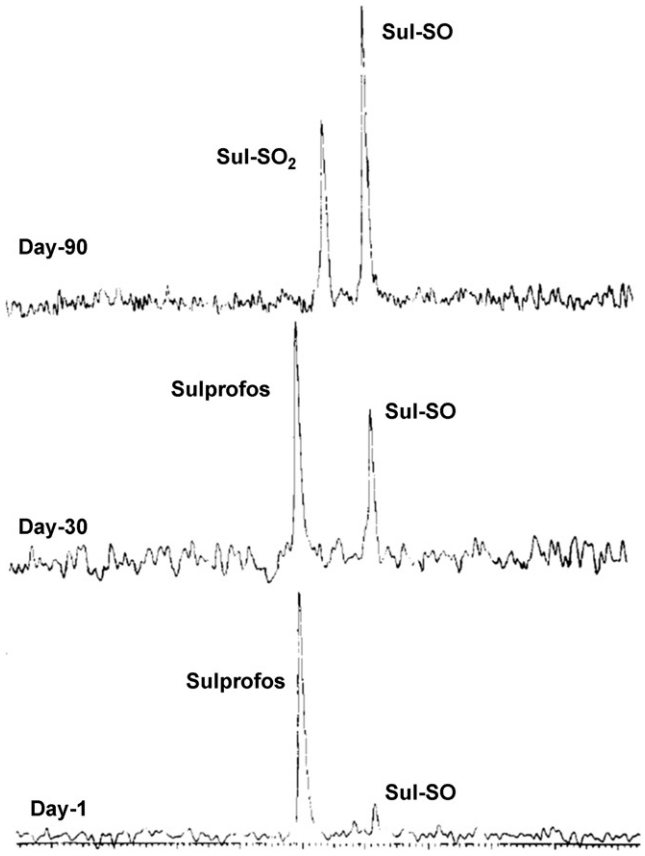

Fig. 9. 121 MHz $^{31}P\{^{1}H\}$ NMR spectra of soil extracts containing sulprofos, and the metabolites sulfoxide (Sul–SO) and sulfone ($Sul–SO_2$).
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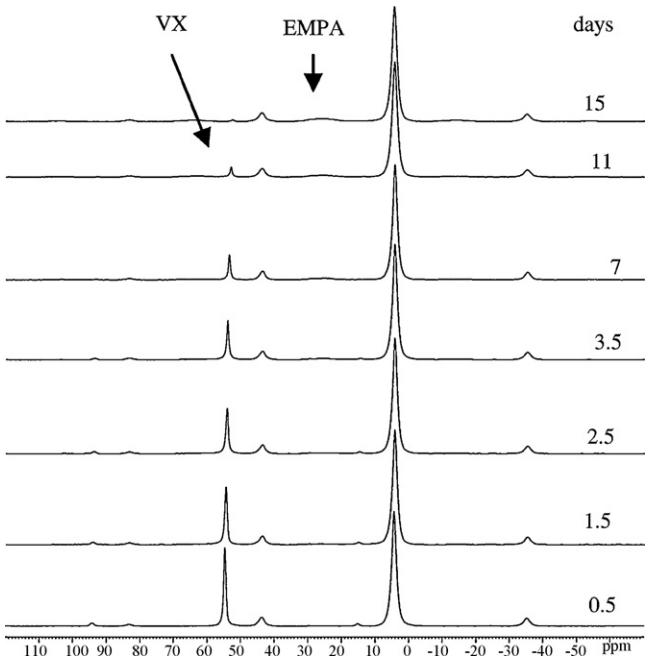

Fig. 10. 202 MHz ^{31}P MAS NMR spectra of desert sand spiked with VX. The peak of the degradation product ethyl methylphosphonic acid (EMPA) is marked with an arrow. The peak from the endogenous phosphate(s) present in the sand is shown at 4 ppm.
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was reported by Mizrahi and Columbus [131]. They monitored the degradation of VX in different urban matrices like concrete, desert sand, beach sand, asphalt, and bitumen sheet (Fig. 10). Based on the results, they were able to define the degradation half-times and propose the matrix-dependent degradation mechanisms for VX.

2.7. Degradation in detoxification solutions

Because of the devastating consequences of chemical warfare considerable resources in military research have been assigned to develop suitable protection against CWA attack. The development has been concentrated on the physical protection of body and respiration, medical protection involving pretreatment and therapy, and detection for alarming, monitoring, identification, and verification of CWA [3] but also on the efficient decontamination of contaminated skin, equipment, vehicles, land areas, and other surfaces [132,133]. Furthermore, the CWC obligates that existing CWA stockpiles and production facilities are destroyed, so those States Parties owing large stocks of CWAs have needed methods for efficient dispose of munitions.

Decontamination can be performed with dedicated detoxification solutions, foams or powders. A typical degradation pathway of the CWA that lowers the toxicity is hydrolysis. The G-series nerve agents like sarin and soman are hydrolyzed in aqueous solutions through nucleophilic substitution of fluorine by hydroxide ion [134]. The V-series nerve agents are more resistant to the aqueous condition, but, for example, VX can undergo autocatalytic hydrolysis in presence of equimolar amount of water [135]. The decontamination solutions are typically formulated from reagents which promote this hydrolysis and other form of degradation, and usual approaches in the optimization of CWA detoxification are the pH control of the solution with strong alkali and the addition of hydrolyzing or oxidizing reagents [136–138].

Yang et al. have made a significant contribution in the CWA detoxification studies during the last decades [132,135,137–139], and have also applied NMR spectroscopy in this field of research. In the review by Yang et al. [132] some examples of NMR characterization were presented in the analysis of CWA degradation in decontamination solutions. Yang [139] also studied the degradation pathways of typical OP nerve agents in several detoxification solutions. In this paper the author stated, that NMR spectroscopy is a useful technique in monitoring CWA reactions in decontamination solutions *in situ*, thus eliminating the need to manipulate the highly toxic samples for analysis, what is needed for gas and liquid chromatographic analyses. NMR spectroscopy can also be performed on concentrated samples typical of neutralization reactions, allowing the observation of interactions between the CWA and degradation products, intermediates, or byproducts which may not exist in the dilute gas or liquid chromatography samples.

Waysbort et al. [140] reported a decontamination system variant of Decon Green™ [141] for CWA detoxification. The decontaminant system, which was composed of a liquid decontamination reagent solution with solid sorbent particles, was tested for reactivity with mustard, soman, and VX. The reaction mixture of contaminated slurry was flame-sealed in a liquid NMR tube, for operator safety. NMR spectroscopy techniques were used to measure the kinetics of the reaction and confirm that the CWA in the matrix was reacted to less toxic products. The two studied OP compounds, soman and VX, were found to degrade rapidly in the proposed decontamination system; reaction half-times under six minutes were reported with almost all modifications of the system.

2.8. Verification analysis

A key feature of the CWC [5] is its extensive verification system. It allows the OPCW [6] to follow the compliance of the States Parties

with the CWC, and by that find possible violations. The OPCW has the mandate for inspections of particular facilities, such as those used to store chemical weapons awaiting destruction, as well as those formerly used for production of chemical weapons. Relevant chemical industry facilities can also be subject to inspections. Routine inspections of chemical industry are focused primarily on those chemicals which pose a risk to the CWC through their potential applications either as CWAs or as precursors for their production. Under the provisions of the CWC, such chemicals may only be produced in very limited quantities for research, medical and protective purposes.

Inspections of a chemical facility may require collection and analysis of samples in order to verify the compliance of the facility to the CWC. The sample can be taken from various materials and matrices found from the inspected site, like aqueous or organic liquid, soil, sand, concrete, paint, and rubber. Inspection procedures allow the preliminary on-site analysis using equipment brought by the OPCW inspection team. Furthermore, the network of the OPCW designated laboratories is capable of conducting consecutive off-site verification analysis from the samples in order to make independent verification of the presence or absence of the chemicals related to the CWC. In order to monitor the competence and analytical readiness of the designated laboratories, the OPCW organizes Proficiency Tests annually in which participating laboratories analyze samples and report any Scheduled Chemicals [5] found. The challenging features of the Proficiency Tests are the low spiking level (1–10 μg/ml or μg/g) and the tight timetable for the analysis [142]. Identification of spiked chemicals, including sample preparation, analysis of the chemicals, and reporting of results must take place within 15 days from the arrival of the samples.

An additional concern of the CWC is the abandoned CWAs. These can be non-adequately disposed nerve agents in munitions and bulk containers. Former production facilities and storage and disposal sites can be contaminated by CWAs. The inspection of such abandoned CWAs can pose risks, as, in addition to CWAs, the munitions can contain undetonated explosives. It is also possible, that munitions contain chemical weapons simulants, or be "dummy" rounds with relatively benign fills that were used for targeting or range-finding. After firing, the munitions may have been buried in for potentially decades of time, the exterior may be corroded to obscure any markings, and no documentation may exist to describe content. As a result, verification analysis is needed to determine the content, and thus give guidance for the proper method for disposal.

All instrumental techniques available are usually employed in verification analysis in order to reliably identify the nature of the sample content. Techniques characterized by high sensitivity, such as gas and liquid chromatography combined with mass spectrometry (LC–MS, GC–MS), have been favored in the verification analysis [143–145]. NMR spectroscopy has played a minor role owing to the modest sensitivity and difficulties associated with the mixture sample analysis. Nevertheless, a major part of the chemicals related to the CWC are OP compounds, which can be screened for in liquid samples with the ^{31}P NMR spectroscopy. Therefore, the ^{31}P NMR spectroscopy has been the cornerstone of the NMR techniques that has been used in the verification analysis. Other one-dimensional (1D) NMR techniques, like ^{1}H NMR, ^{19}F NMR and ^{13}C NMR, have also been used when applicable.

The Ministry for Foreign Affairs of Finland published between 1977 and 1994 a series of "Finnish Blue Books" [146] that contained systematic test results and recommendations for sampling and analysis of chemicals related to the CWC. Enqvist et al. [147] described in one of the books a standardized verification method that included also ^{31}P NMR spectroscopy as an important screening and identification technique for typical OP nerve agents. In the last book of the series [148], the recommended operating procedure for identification of the CWC-related compounds by NMR spectroscopy was given.

Wils et al. [149] presented a summary of an international round-robin verification exercise where the samples from a simulated facility inspection with alleged production of CWA where analyzed by two laboratories. The scenario of this exercise was to verify that the facility, which normally produced OP insecticide dichlorvos did not have any production of OP compounds that are related to the CWC. The simulated samples from the feedstock chemical storages, reaction vessel, and auxiliary feedline were analyzed with GC–MS and NMR. ^{19}F NMR spectroscopy was used as a complementary technique to verify the presence of sarin and isomeric mixture of octyl methylphosphonofluoridates, which are Scheduled Chemicals in the CWC [5]. In addition, dioctyl methylphosphonate, octyl methylphosphonic acid, and methyl octyl methylphosphonate were detected from the simulated samples with ^{31}P NMR spectroscopy.

In connection with the first Trial Proficiency Test, Mesilaakso and Tolppa reported the sample preparation, NMR experiments, and identification by NMR of compounds relevant to the CWC that were spiked in paint, rubber, and soil matrices [150]. The following paper from Mesilaakso [151] describes the ^{1}H NMR and ^{31}P NMR analyses of organic extract, water, and sand samples for the identification of the Scheduled Chemicals and their degradation products. The general NMR analysis strategy of the CWC-related chemicals has been proposed by Mesilaakso and Niederhauser [152,153].

Creasy et al. [154] applied NMR among other instrumental techniques (GC–MS, GC–AED, CE, LC–MS) in analysis of CWA decontamination waste in old ton containers from Johnston Atoll. ^{31}P NMR, ^{1}H NMR, and ^{13}C NMR were used on selected samples to confirm the chemical identification, determine their distribution in the samples, and search for any other relevant chemicals that could be missed by other methods. The ton containers were originally used to dispose decontamination waste of sarin, VX and mustard gas. In this case study the level of effort required to characterize other types of bulk waste was assessed for future CWA-related cleanups. The authors found that none of the samples contained residual sarin or VX above the detection limit 20 ng/ml, and the only OP compounds found were the typical degradation products of sarin and VX, namely isopropyl methylphosphonic acid, ethyl methylphosphonic acid and methylphosphonic acid. It was reported, that ^{31}P NMR was particularly important in confirmation of ethyl methylphosphonic acid, as it was not always detected by GC–MS due to difficulties in derivatization. However, iron and iron oxide found in some of the samples required additional filtering or use of a complexing agent in order to prevent the broadening of the NMR peaks.

Borrett et al. [155] studied amiton (*O*,*O*-diethyl-*S*-[2-(diethylamino)ethyl] phosphorothiolate) and its degradation products in concrete, paint, rubber and soil matrices. Amiton was developed in 1950s as an insecticide, but because of its high toxicity it has been included in the CWC as a Scheduled Chemical. Their main interest was to test suitable sample preparation and derivatization methods for GC–MS based verification analysis, but ^{31}P NMR was also applied in the analyses as a complementary identification technique of the degradation products.

While 1D NMR, ^{31}P NMR in particular, has been the typical NMR technique in CWA analysis, traditional 1D NMR methods are not always adequate in detection of traces of CWAs and related OP compounds. ^{31}P NMR spectroscopy offers a selective way to detect relevant OP compounds, but the information content of the spectrum may be inadequate to distinguish similar OP compounds from each other. On the other hand, intense signals from the main components of the solution matrix cause problems in ^{1}H NMR analysis and can mask resonances of the chemicals of interest. In these cases more modern NMR methods can offer several benefits. 1D and 2D

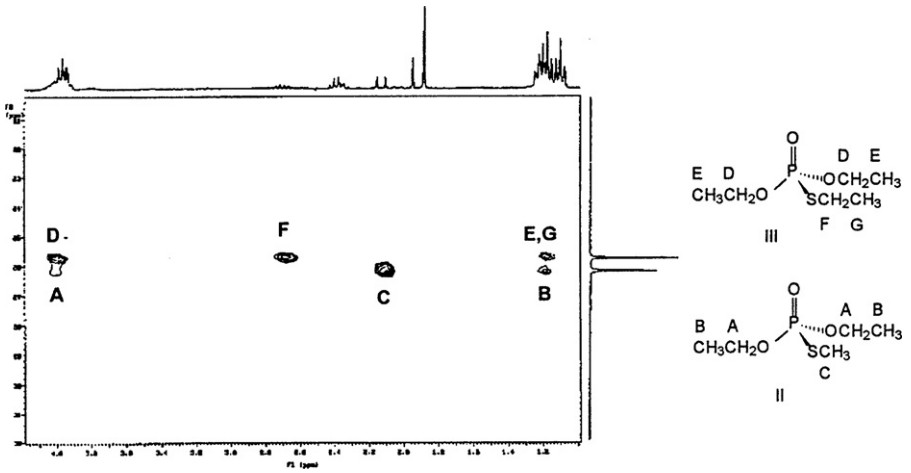

Fig. 11. 121 MHz 2D ^{31}P–^{1}H HETCOR spectrum used to identify compounds *O*,*O*-diethyl *S*-methyl phosphorothioate (II) and *O*,*O*,*S*-triethyl phosphorothioate (III) in the munition.
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multipulse experiments [156], which are routinely used in organic chemistry for characterization of synthesis products, have also been taken into use in the verification analyses. Brickhouse et al. [157] applied several instrumental techniques in verification analysis of a munition shell that contained an amber yellow organic liquid. The preliminary GC–MS analysis indicated presence of trace levels of VX and a commercial pesticide cyanthoate (*S*-[2-[(1-cyano-1-methylethyl)amino]-2-oxoethyl] *O*,*O*-diethyl phosphorothioate). The applied NMR experiments included ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, DEPT-135 [158], ^{1}H–^{1}H COSY [159], ^{13}C–^{1}H HETCOR [160] and ^{31}P-^{1}H HETCOR [161]. In particular, the ^{31}P–^{1}H HETCOR proved to be useful in characterization of two of the OP compounds present in the sample (Fig. 11). Finally, six high-concentration OP compounds were identified and confirmed by GC–IR–MS, LC–MS, GC–AED, and NMR. Two major phosphorus compounds found in the sample were *O*,*O*,*O*-triethyl phosphorothioate and *O*,*O*,*S*-triethyl phosphorothioate; no VX, G-agent, or pesticide were observed. The authors concluded that the mixture appears to have been formulated as a chemical warfare agent simulant, most likely as a challenge to agent detection techniques.

Lower sensitivity of phosphorus compared to proton can hinder the application of phosphorus-detected two-dimensional

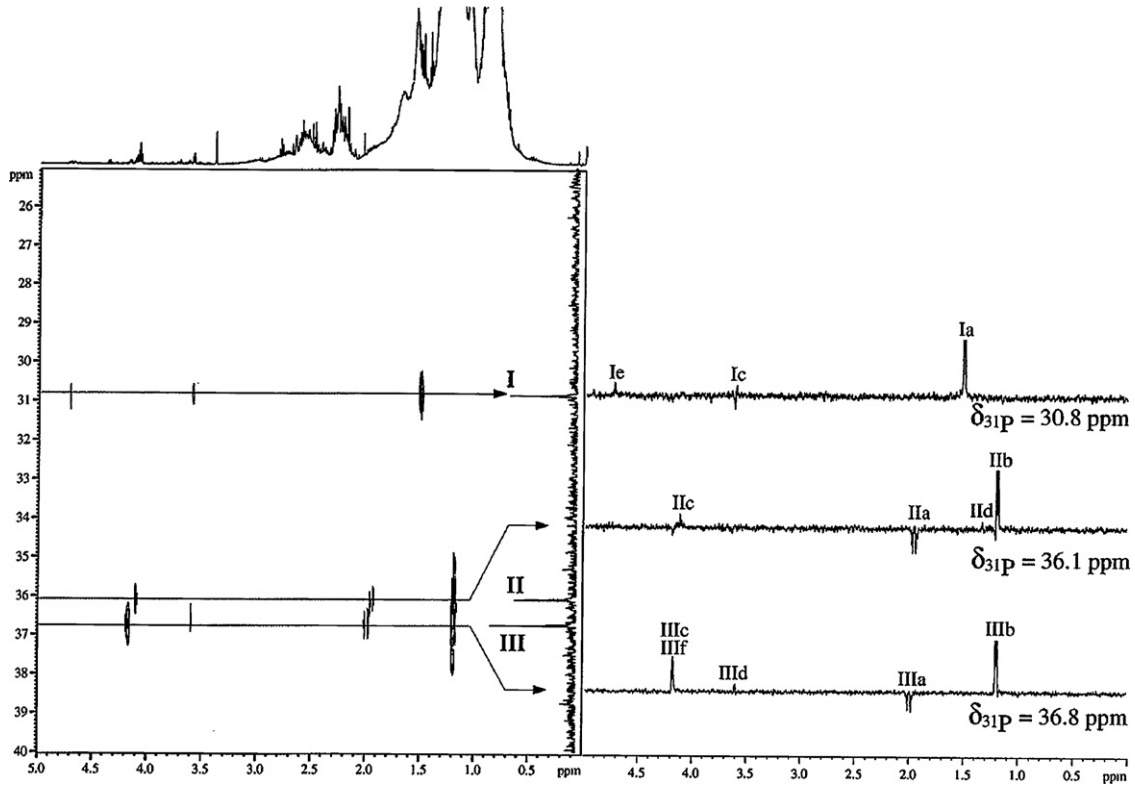

Fig. 12. 500 MHz 2D ^{1}H-^{31}P HSQC spectrum and F_2 traces used to identify compounds (2-methoxyethyl)isopropyl methylphosphonate (I), diethyl isopropylphosphonate (II) and (2-methoxyethyl)ethyl isopropylphosphonate (III) in the sample prepared from the 2nd OPCW Proficiency Test soil sample. The proton resonances with J_{PH} are shown in the spectrum, while the background resonances shown in the proton spectrum (top projection) are eliminated. The total acquisition time was 4 h 25 min.
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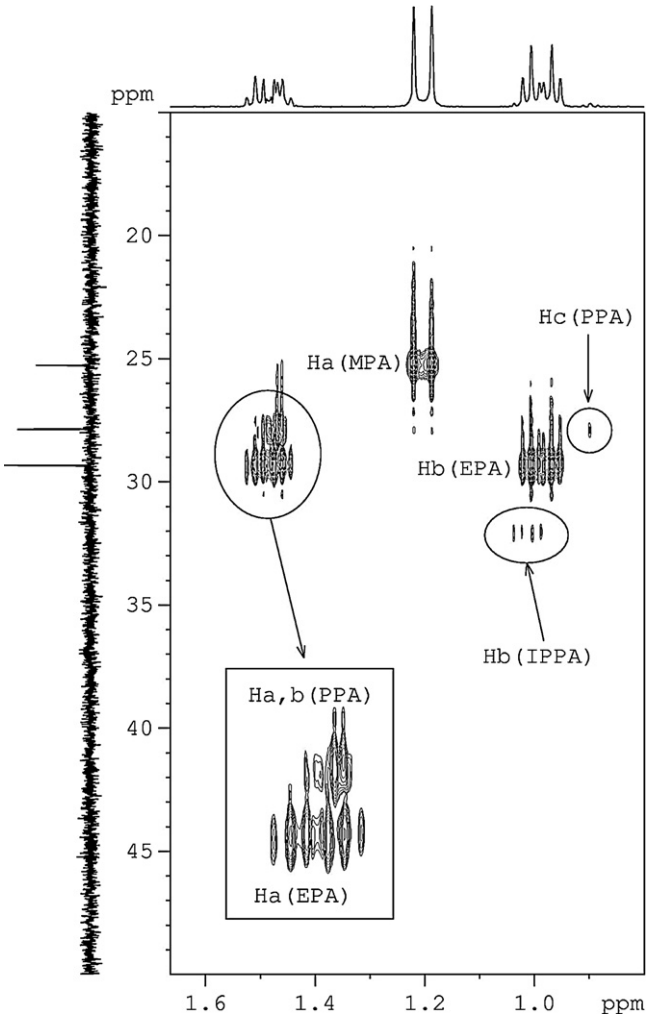

Fig. 13. Expansions of 500 MHz 2D ^{1}H-^{31}P fast-HMQC spectrum from a sample containing methylphosphonic acid (MPA) ethylphosphonic acid (EPA), propylphosphonic acid (PPA), and isopropylphosphonic acid (IPPA) at levels 6.7, 10.0, 8.9, and 0.3 μg/ml, respectively. The F_2-skyline projection of the fast-HMQC spectrum and the 202 MHz ^{31}P{^{1}H} NMR spectrum are plotted along the F_2 and F_1 axes, respectively. IPPA, which is present at sub-parts-per-million level (0.3 μg/ml), can not be seen in the ^{31}P{^{1}H} NMR spectrum, but the cross-peak of Hb protons of IPPA is detectable in the 2D ^{1}H-^{31}P fast-HMQC spectrum. The total acquisition time of 2D ^{1}H-^{31}P fast-HMQC and ^{31}P{^{1}H} NMR spectra were 3 h 40 min and 5 h 30 min, respectively.
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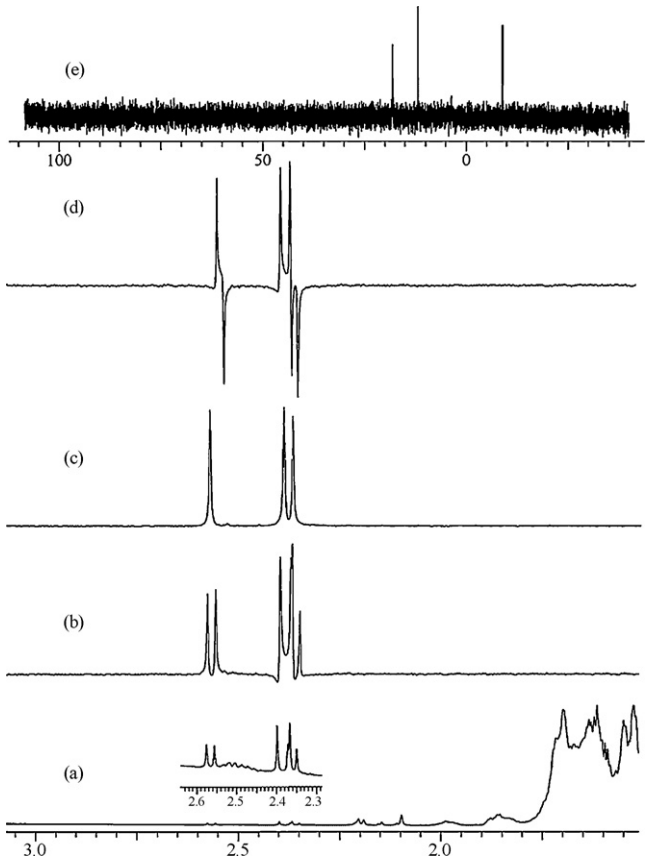

Fig. 14. 500 MHz ^{1}H NMR (a), 1D ^{1}H-^{31}P HSQC (b), 1D ^{1}H-^{31}P HSQC with ^{31}P decoupling (c), 1D ^{1}H-^{31}P HSQMBC (d), and 202 MHz ^{31}P{^{1}H} NMR (e) spectra from the 1st OPCW Proficiency Test organic liquid sample containing 37.5 μg/ml ethyl *N*,*N*-dimethylphosphoramidocyanidate, 37.5 μg/ml diethyl *N*,*N*-dimethylphosphoramide, and 37.5 μg/ml (dimethylamino)phosphoryl dichloride. The background produced by the diesel oil dominates the ^{1}H NMR spectrum (a). In the (b), (c), and (d) spectra background signals due to the diesel oil are completely removed, and the proton signals from the relevant OP compounds are clearly seen. Acquisition of the proton-detected spectra was reported to took 15 min, while the acquisition of ^{31}P{^{1}H} NMR spectrum (e) took 62 min, thus demonstrating the sensitivity advantage of the inverse detection of phosphorus with respect to the direct observation.
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correlation experiments in trace level analyses. Inverse-detected two-dimensional ^{1}H-^{31}P correlation experiments, on the other hand, offer the advantages of the both nuclei, i.e. sensitivity of proton and selectivity of phosphorus. An example of the application of 2D ^{1}H-^{31}P HSQC (heteronuclear single quantum coherence) [162] experiments in the OPCW Proficiency Test analysis was presented in 1997 by Albaret et al. [163]. The soil sample that was analyzed in the test contained three OP compounds that were relevant to the CWC (Fig. 12). Acquisition of 2D ^{1}H-^{31}P HSQC spectrum was reported to take under 5 h with a sample containing 50–100 μg/ml of the OP compounds.

In some cases a higher sensitivity is desired. One possibility to improve the sensitivity of 2D correlation experiments is with the Ernst angle optimization technique proposed by Ross et al. [164]. This 2D ^{1}H-^{31}P fast-HMQC approach [165] can facilitate the detection of even sub-parts-per-million levels of OP compounds within reasonable total acquisition time (Fig. 13).

Sometimes screening of the sample for OP compounds without further characterization of the structures is sufficient. In those cases the one-dimensional proton-phosphorus correlation experiment can offer a rapid confirmation of the presence or absence of the OP compounds. Meier [166] demonstrated with 1D ^{1}H-^{31}P HSQC and 1D ^{1}H-^{31}P HSQMBC (heteronuclear single quantum multiple bond correlation) [167] experiments that screening of solutions for the presence of OP compounds related to the CWC is much faster with inverse-detected 1D ^{1}H-^{31}P correlation experiments compared to ^{31}P NMR spectroscopy (Fig. 14).

The 1D ^{1}H-^{31}P HSQC experiment is also useful in rapid screening of OP compounds in the analysis of untreated decontamination solutions [168]. The degradation products of sarin, soman, and VX can be detected at level 2–10 μg/ml with half an hour acquisition. Furthermore, the method is also suitable, on certain condition, for the quantification of OP compounds.

The high level of background chemicals poses a problem in the NMR analysis. Since the seminal work of Watanabe and Niki [169] the hyphenation of liquid chromatography with NMR (LC–NMR) [23] has intrigued analytical chemists due to its potential in analysis of complex samples, and various techniques have been developed [170]. Though LC–NMR has been applied with success in several

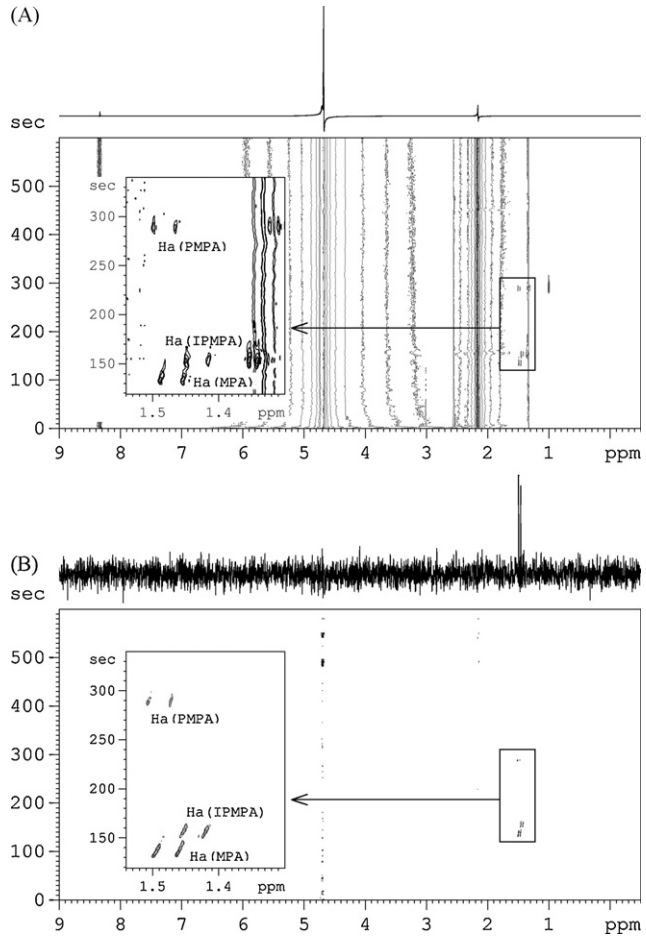

Fig. 15. 500 MHz pseudo-2D spectra from on-flow ^{1}H NMR acquisition with two-band WET [179] suppression on water and acetonitrile peaks (A) and on-flow 1D ^{1}H-^{31}P HSQC acquisition (B) of 20 μl injections of a sample containing 10 μg of methylphosphonic acid (MPA), isopropyl methylphosphonic acid (IPMPA), and pinacolyl methylphosphonic acid (PMPA), characteristic degradation products of sarin and soman. Flow rate was 1 ml/min of 30% acetonitrile and 70% water (with 0.1% formic acid). Both spectra are plotted close to the noise level. On top of pseudo-2D spectra are the observed 1D spectra at the MPA retention time.
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fields of research, applications in the identification of CWC-related chemicals have been rare. Probably the first mention about the CWA analysis with LC–NMR is by Preiss and Godejohann [171]. They briefly described potential of LC–NMR in analysis of the 6th OPCW Proficiency Test soil sample using on-flow ^{1}H NMR. Identification of the relevant resonances of OP compounds was facilitated with ^{31}P decoupling. Solid phase extraction (SPE) in conjunction with LC–NMR (LC–SPE–NMR) [172] is another technique that can be used for selective enrichment of the selected analytes prior to the NMR analysis. The problem with the OP chemicals is their lack of chromophore, so their detection during chromatographic separation needs consideration. One possibility is to apply on-flow 1D ^{1}H-^{31}P HSQC [173] for the selective detection of characteristic OP degradation products during chromatographic separation. This approach offers a high dynamic range and good detection limit (ca. 10 μg/55 nmol in 20 μl injection) with a high sampling frequency (1 point per 2 s, flow rate 1 ml/min) in the acquired pseudo-2D spectrum (Fig. 15). After the retention time determination, SPE enrichment, and elution to capillary NMR tubes, OP compounds were separately identified off-line using a mass-sensitive microcoil probe head [174].

Mazumder et al. [175] demonstrated the use of the on-line LC–UV–NMR technique in the CWC-related analysis. They applied derivatization of selected phosphonic acids relevant to the CWC with phenyl diazomethane [176] in order to improve retention of the OP compounds in chromatographic separation. Additionally, the derivatization facilitated UV detection of the otherwise non UV-active OP compounds giving additional identification. The authors applied on-flow ^{1}H NMR with WET for the identification and reported the detection limit of 16 μg in 20 μl injection with the sampling frequency of 1 point per 150 s (flow rate 0.1 ml/min) in the acquired pseudo-2D spectrum. The drawback of the on-flow ^{1}H NMR was the loss of the some of OP compounds proton resonances in the vicinity of the suppressed solvent signals, complicating the identification.

3. Conclusion

NMR spectroscopy has played an important role in many aspects of the OP compounds analysis. The main advantage of the technique is that it gives information about the structure, interactions, and reactions of OP compounds. Other valuable features of NMR are that it is quantitative, nondestructive, and it simultaneously detects all components of a mixture. The modest sensitivity of NMR has hindered the trace analysis of OP compounds, but the recent advances in the probehead [177] and hyperpolarization [178] technologies may change this in the future. Due to its simplicity, ^{31}P NMR spectroscopy will hold its position as the main NMR technique in OP compounds analysis, but the 2D NMR spectroscopy which offers improved resolution of the peaks can be expected in the future to be more common in the studies of OP compounds in mixture samples. The hyphenated LC–NMR and LC–SPE–NMR techniques are more demanding, but with a little effort the system can be optimized for the given task and by that further facilitate the identification of the chemicals in mixture samples.

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