Application of microcolumn liquid chromatography and capillary electrophoresis with flame photometric detection for the screening of degradation products of chemical warfare agents in water and soil

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Abstract

Microcolumn liquid chromatography (μLC) and capillary electrophoresis (CE) coupled on-line with flame photometric detection (FPD) have been used for the screening of polar breakdown products of chemical warfare agents in water and soil samples, provided during Official Proficiency Tests organized by the Technical Secretariat of the Organization for the Prohibition of Chemical Weapons. CE–FPD is shown to be a powerful and rapid method for the determination of alkylphosphonic acids, which are the breakdown products of organophosphorus nerve agents. Gradient elution μLC–FPD is more sensitive and robust but less rapid in the determination of these compounds. In addition, μLC–FPD can be applied to screen for hydrolysis products of sulfur mustard and its analogues. Both methods can be applied without prior derivatization and are extremely selective. In order to unambiguously identify the relevant compounds, electrospray ionization (tandem) mass spectrometry, gas chromatography–mass spectrometry and nuclear magnetic resonance spectrometry were applied. © 2001 Elsevier Science B.V. All rights reserved.

1. Introduction

Since the Chemical Weapons Convention (CWC) entered into force in 1997, many efforts have been made to develop and improve methods for the determination of chemical warfare agents (CWAs) and their precursors or degradation products. Verification analysis of this treaty, which prohibits the development, production, stockpiling and use of CWAs, is an important component of monitoring compliance with the CWC.

Most CWAs are rapidly hydrolysed in the presence of water to more persistent degradation products. Generally, these hydrolysis products are highly polar and frequently they are non-volatile and/or do not

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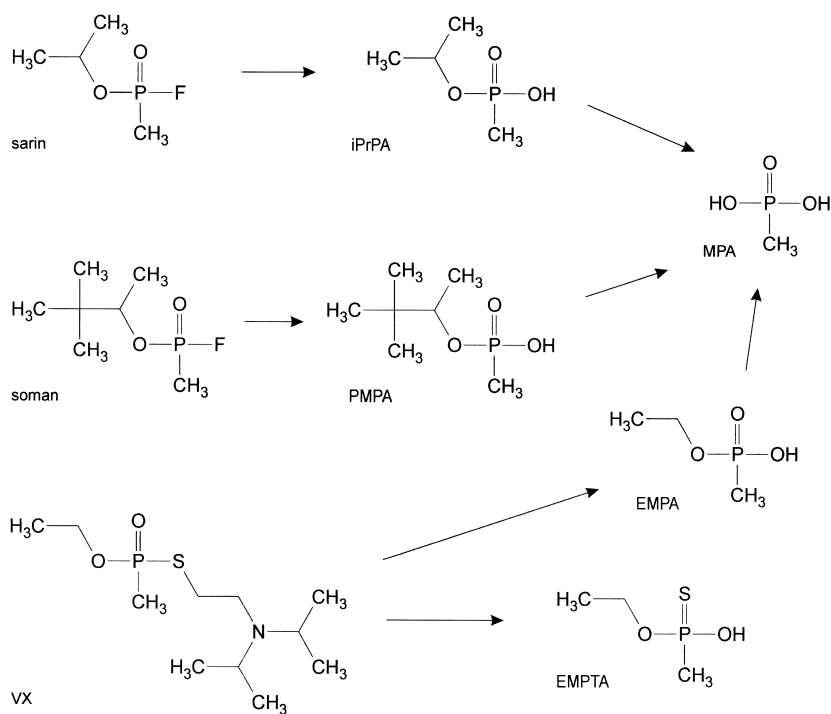*E-mail address:* ehooijschuur@pbr.nl (E.W.J. Hooijschuur).

Fig. 1. Hydrolysis pathways of organophosphorus nerve agents. For acronyms, See Section 2.1.

contain chromophoric groups. Relevant examples for the two main classes of CWAs are given in Fig. 1: nerve agents rapidly hydrolyse to alkylphosphonic acids, and Fig. 2: blister agents, the sulfur mustards, are easily converted to thiodiglycol and its oxidation products, or bis(2-hydroxyethylthio)alkanes and their oxidation products.

The most frequently used methods for the unambiguous identification of the non-volatile highly polar hydrolysis products of nerve agents as well as the highly polar degradation products of mustards in aqueous samples are based on gas chromatography (GC) in combination with mass spectrometry (GC–MS) and/or tandem mass spectrometry (GC–MS–MS) [1–3]. GC coupled with Fourier transform infrared spectrometry (GC–FTIR) [4] has also been used. However, all GC-based methods require sample preparation and derivatization prior to analysis. In addition, derivatization may lead to the formation of artifacts and may cause low recoveries due to the presence of interfering compounds. Recent developments based on rapid hyphenated methods such as column liquid chromatography (LC) and capillary

electrophoresis (CE) coupled with a variety of (tandem) MS detection techniques [5–7] are therefore of great importance for identification purposes.

Separation techniques such as GC, LC and CE can also play an important role as preselection method when large numbers of samples have to be analysed. They are also essential for the determination of known CWAs and related products in all types of sample. GC with flame photometric (GC–FPD) [8,9] as well as atomic emission (GC–AED) detection [10] have been used for the determination of non-volatile highly polar hydrolysis products. However, these methods are better suited to screen for the presence of intact agents or related compounds that are relatively volatile, i.e. when no time-consuming derivatization has to be carried out. In so-called 'alleged use' investigations, and especially when many samples are involved, the time delay may become unacceptably long. LC and CE are better suited to handle the non-volatile highly polar hydrolysis products. CE, with indirect UV [11] or conductivity [12] detection, is a suitable method for the rapid screening of alkylphosphonic acids; with
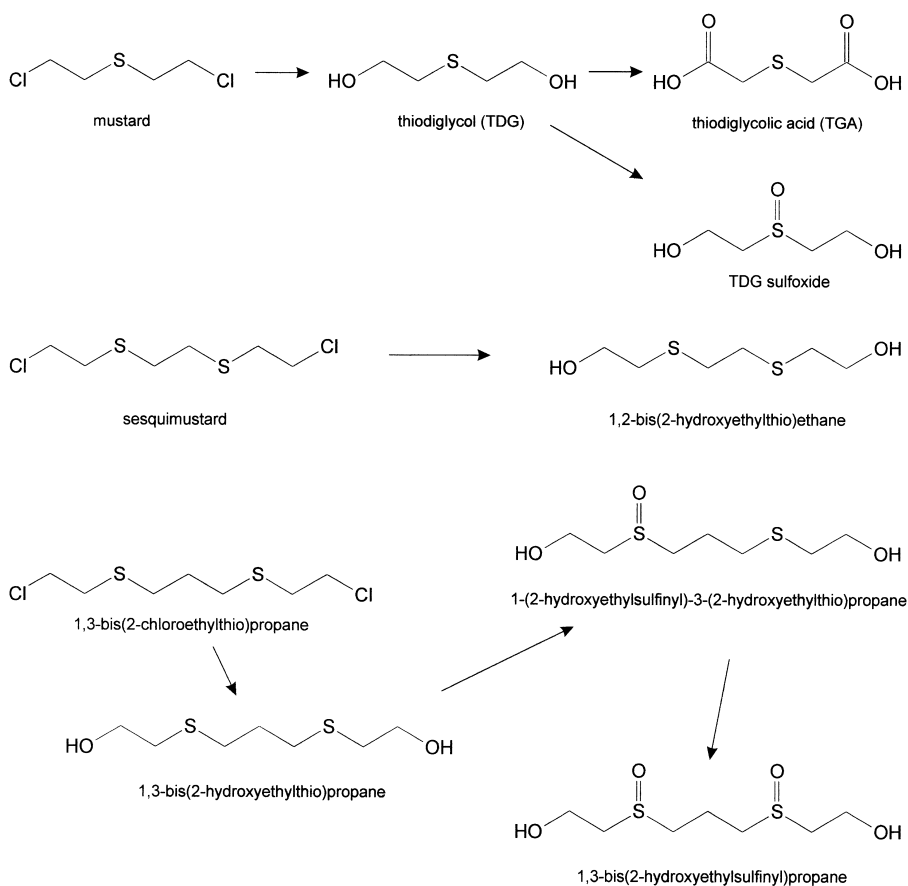
Fig. 2. Hydrolysis and oxidation pathways of sulfur mustards.

aqueous buffer systems there is no need of an organic additive and sample pre-treatment is minimal. Ion chromatography (IC) and LC coupled with evaporative light scattering detection (ELSD) can also be used for the determination of alkylphosphonic acids [13,14]. Micellar electrokinetic chromatography (MEKC) with UV detection has been applied for the screening for S-containing CWA-related compounds [15]. Our group developed a number of methods using μLC and CE coupled on-line with FPD in the P-mode [16,17], and μLC–FPD in the S-mode [18,19]. These techniques enable the direct, rapid and selective determination of degradation products of nerve and mustard agents.

To test the ability of participating laboratories to unambiguously identify compounds relevant to the CWC, the Technical Secretariat of the Organization for the Prohibition of Chemical Weapons (OPCW) has, up to the year 2000, organized eight Official Proficiency Tests. Samples spiked with relevant chemicals at a concentration level of at least 1–10 μg/g, included water, soil and organic liquids; corresponding blank samples were also provided. Emphasis was on the unambiguous identification rather than the quantification of the compounds of interest. After receipt of the samples, 15 days were allowed for analysis and reporting. For unambiguous identification of a compound, at least two different analytical methods giving consistent results were required.

In this paper, the practicality of the earlier reported μLC–FPD and CE–FPD methods as demonstrated during three Official Proficiency Tests is discussed. Since these screening procedures are

preliminarily used to verify the presence of polar degradation products of CWAs, only those samples that were expected to contain such compounds, viz. water and aqueous extracts of soils, were analysed. The results obtained with these techniques are presented along with nuclear magnetic resonance (NMR) and mass spectrometric data, viz. GC–MS and electrospray (ESI) LC–MS, that lead to the unambiguous identification of the CWC-related compounds in the samples provided.

2. Experimental

2.1. Materials

Analytical-grade formic acid, 25% ammonia solution, methanol and phosphoric acid (PA) were purchased from Merck (Darmstadt, Germany). Ammonium formate, ammonium acetate, methylphosphonic acid (MPA), ethylphosphonic acid (EPA), *n*-propylphosphonic acid (nPrPA), isopropylphosphonic acid (iPrPA), *n*-butylphosphonic acid (nBPA) and phenylphosphonic acid (PhPA) were obtained from Aldrich (Milwaukee, WI, USA); thiodiglycol (TDG) and trimethyl phosphate were from Sigma (St Louis, MO, USA). Ethyl methylphosphonic acid (EMPA), ethyl methylthiophosphonic acid (EMPTA), methyl ethylphosphonic acid (MEPA), isopropyl methylphosphonic acid (iPrMPA), isopropyl ethylphosphonic acid (iPrEPA), pinacolyl methylphosphonic acid (PMPA), dimethyl ethylphosphonate (DMEP), dimethyl phenylphosphonate (DMPhP) and 1,5-bis-(2-hydroxyethylsulfinyl)pentane were synthesized at TNO Prins Maurits Laboratory (TNO–PML). Throughout the study, deionised water (Milli-Q water Purification System; Millipore, Milford, MA, USA) was used. All solvents and solutions were filtered prior to use over 0.45-µm pore size filter disks from Alltech (Breda, The Netherlands).

2.2. Instrumentation

2.2.1. CE–FPD and µLC–FPD

For the CE–FPD as well as the µLC–FPD set-up, an FPD Model 380 (CE Instruments, Milan, Italy) was used. The detector flow-rates were 300 ml/min (air), 600 ml/min (hydrogen) and 40 ml/min (helium). The interface used for both set-ups is described in detail elsewhere [16]. Fused silica capillaries of different diameters were obtained from Composite Metal Services (Hallow, UK) and Supelco (Bellefonte, PA, USA), and PEEK tubing from Alltech. All experiments were performed at ambient temperature.

Data collection and processing was performed using Xchrom or Atlas 98 (LabSystems, Altrincham, UK). The data-sampling rate was set at 6.25 Hz.

2.2.2. Set-up of the CE–FPD system

For CE–FPD, a PRINCE CE system including a UV detector (Prince Technologies, Emmen, The Netherlands) was used. The CE–FPD set-up is described in detail elsewhere [17]. Untreated fused-silica separation capillaries (75 cm×50 µm I.D., 375 µm O.D.) were used, except where indicated. The applied voltage was +30 kV in all experiments. The separation buffer was a 50 m*M* ammonium acetate solution adjusted to pH 9.0 with ammonia, except where indicated. The separation capillary outlet was inserted in a Knurl-Lok III PEEK T-piece (Alltech) together with PTFE tubing (Alltech) which was used to deliver a post-column make-up flow of 0.5% formic acid, induced by a hydrostatic pressure (ca. 300 mbar) that was used to ground the CE system and to achieve a suitable flow-rate (ca. 10 µl/min) for the interface. An introduction capillary (40 cm× 100 µm I.D., 170 µm O.D.) led from the T-piece through the interface into the flame of the FPD. The make-up pressure was counterbalanced at the inlet of the capillary to eliminate a pressure-driven flow in the CE capillary. During injection, no make-up pressure was applied. The various stages of the on-column sample concentration procedure used to enhance the analyte detectability in CE–FPD, are summarized in Table 1.

2.2.3. Set-up of the µLC–FPD system

Briefly, a Phoenix 20 CU (CE Instruments) syringe pump and a Phoenix 20 CU slave pump were connected via a Valco mixing chamber (VICI, Schenkon, Switzerland) with a Valco six-port valve having a variable external injection loop of PEEK tubing. The six-port valve was connected to a Valco micro-injection valve with a 60-nl internal volume. Fused silica columns were slurry-packed at TNO–PML

Table 1
On-column sample concentration procedure for CE–FPD

Step	Procedure
1	Capillary is rinsed with water, 0.1 M NaOH, water (1000 mbar, 2 min each); no make-up pressure is applied
2	Capillary inlet is inserted in CE buffer vial, capillary is filled with CE buffer (1000 mbar, 5 min); simultaneously, PTFE tubing is filled with make-up solution (make-up pressure 300 mbar)
3	Make-up pressure is released, capillary inlet is inserted in sample vial, 300 nl of sample are introduced (350 mbar for 0.7 min)
4	Capillary inlet is inserted in CE buffer vial. Reversed electroosmotic flow directed to capillary inlet is induced ($-10/-15$ kV, 3–6 min): sample matrix is directed to CE buffer vial; make-up solution enters capillary. Simultaneously, the analytes are focused at cathodic end of capillary (stacking effect); consequently, none of the analytes of interest is lost
5	CE is started ($+30$ kV, 16 min). Simultaneously, make-up pressure and pressure at capillary inlet are applied (300 mbar)

with 10 μm PRP-X100 (Hamilton, Reno, NE, USA) polymer (18.5 cm×320 μm I.D., 450 μm O.D.) and with 5 μm LiChrosorb RP-18 (Merck) bonded silica (15 cm×320 μm I.D., 450 μm O.D.). The column outlet was inserted in a laboratory-made PEEK T-piece together with the outlet of a Phoenix 20 CU make-up pump via a Valco microinjection valve with a 60-nl internal volume to allow flow-injection optimization of the interface and detector. An introduction capillary (25 cm×100 μm I.D., 170 μm O.D.) led from the T-piece via the interface into the flame of the FPD. For more details, one should consult Ref. [20].

2.2.4. ESI–MS–MS, GC–MS and NMR

Flow injection–ESI-MS–MS was carried out on a Quattro II triple-quadrupole instrument (Micromass, Altrincham, UK) (OPCW tests 2 and 4) or a Q-TOF (Micromass) (OPCW test 6) with a standard ESI interface using a 20–50 V cone voltage in the positive or negative mode, and using argon as the collision gas at an indicated collision energy of 13–20 eV. The eluent consisted of water with 0.2% formic acid–acetonitrile (50:50, v/v) and was provided at a flow-rate of 20 μl/min by a Waters (Milford, MA, USA) M590 solvent delivery system.

GC–MS was carried out using fused-silica capillary columns (50 m×0.32 mm I.D., d_f 0.25 μm) coated with CPSil5CB (Chrompack, Middelburg, The Netherlands) on a HP 5890A or HP 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with an on-column injector (CE Instruments). The column was directly connected to the ion source of a VG70-250S (Micromass) (OPCW

tests 2 and 4) or a Quattro II mass spectrometer (Micromass) (OPCW test 6). The temperature programmes were dependent on the samples analysed; helium at 1.5 ml/min was used as carrier gas. Electron impact (EI, 70 eV) ionization mass spectra were scanned over a mass range of typically m/z 25–500 every second.

^{1}H-NMR (400 MHz) and 162 MHz ^{31}P-NMR spectra were recorded at 30°C using a VXR 400S spectrometer and a Sun OS 5.4 operating system (Varian, Fort Collins, CO, USA). The line widths were typically 0.5–1 and 5–10 Hz for the ^{1}H-NMR and ^{31}P-NMR spectra, respectively.

2.3. Pretreatment of water and soil samples

As the first step, samples were removed from the refrigerator and allowed to warm to ambient temperature. Water samples were analysed without any treatment. Soil samples were extracted according to a standard operation procedure developed in-house. Briefly, 10 g of soil were extracted with 10 ml of Milli-Q water. After 10 min of ultrasonication the liquid was decanted into a centrifuge tube. An additional 10 ml water were added to the soil and after 10 min of ultrasonication decanted into another centrifuge tube. The extracts were centrifuged for 10 min at 10 000 rev./min and, subsequently, combined. When CE analysis was applied, water samples and the aqueous soil extracts were pressed through Bond-Elut SCX cartridges (Analytichem, Harbor City, CA, USA) in order to remove cations. Prior to injection, all samples and extracts were filtered through 0.45-μm pore size filter disks (Alltech).

3. Results and discussion

3.1. CE–FPD of water and soil samples

During the Second Official OPCW Proficiency Test (1996), a spiked water sample and an aqueous extract of a soil sample as well as the corresponding blanks were subjected to CE–FPD. To enable screening of the samples for the presence of alkylphosphonic acids at the required 1 μg/ml level, on-column sample concentration had to be used to reach this concentration limit of detection (LOD) without a serious loss in separation efficiency [17]. Large-volume injections and subsequent removal of the matrix and sample stacking, as described under the Experimental section, were used to this end. However, direct hydrodynamic introduction of a 300-nl volume of the filtered water sample into the system, followed by a matrix removal step (−10 kV for 5.0 min) produced zones instead of peaks of the analytes of interest. To reduce the conductivity of the sample and, consequently, enhance the stacking of the analytes of interest during matrix removal, the sample was pressed through an SCX column in order to remove cations prior to injection. Subsequently, 300 nl were hydrodynamically injected, followed by matrix removal at −10 kV for 3.0 min. Fig. 3 shows the resulting electropherogram (trace B) which is compared with a 12.3-nl injection of an aqueous reference solution containing 50–150 μg/ml of the alkylphosphonic acids, MPA, EMPA, iPrMPA, PMPA, EPA, nPrPA, nBuPA, PhPA and EMPTA and the neutral phosphonate, DMPhP, which was added as an electroosmotic flow marker (trace A). Two P-containing peaks were observed in the trace of the water sample which were absent in the blank (trace C). From the migration times of the reference compounds, PMPA (7.65 min; seven carbon atoms) and iPrMPA (8.40 min; four carbon atoms), it was tentatively concluded that the first unknown (compound **1**), with a migration time of 7.80 min, could be an alkylphosphonic acid having five or six carbon atoms. The second peak (compound **2**) had a rather large migration time (12.20 min) which indicated it to be a divalent anion at this pH. The migration time was rather close to that of EPA (12.05 min; pK_{a1}, 2.45, pK_{a2}, 7.85). Standard addition of 20 μg/ml EPA to the water sample caused a twofold increase
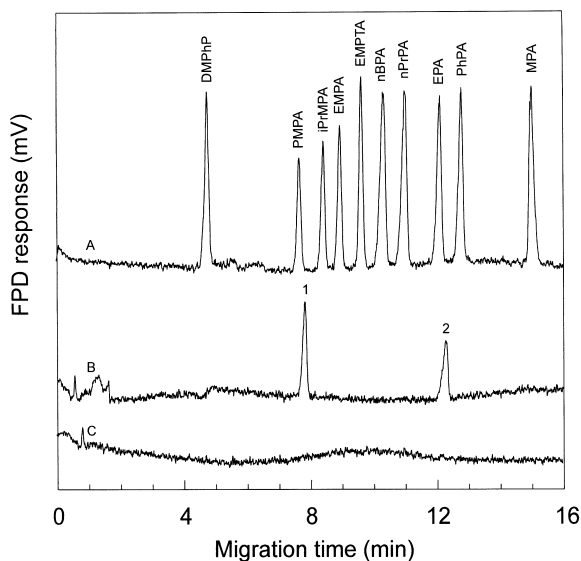

Fig. 3. CE–FPD (P-mode) electropherograms of (A) a 12.3-nl injection of a reference solution (50–150 μg/ml); (B) 300-nl injection of water sample; (C) 300-nl injection of a blank. Separation buffer, 50 mM ammonium acetate (pH 9.0); voltage, +30 kV; make-up, 0.5% formic acid. Matrix removal of (B) and (C), −10 kV for 3.0 min. For further experimental conditions, see text.

in the height of the second peak, giving evidence that compound **2** was most probably EPA. If it is assumed that the spiking was based on an essentially real-life situation where one particular nerve agent was used or produced, both compounds should be breakdown products of that same nerve agent. On the basis of this consideration and the provisional identification of EPA, compound **1** in the electropherogram was tentatively identified as a propyl or butyl ethylphosphonic acid.

The unambiguous identification of EPA, and of compound **1** as isopropyl ethylphosphonic acid (iPrEPA) was done with flow-injection–ESI-MS–MS and GC–EI-MS (after evaporating 1 ml of the sample to dryness followed by *tert*.-butyldimethylsilyl (TBDMS) derivatization) as well as ^{1}H-NMR and ^{31}P-NMR (after evaporating of 10 ml of the sample to dryness, extraction of the residues with deuterated chloroform, evaporation of the chloroform to dryness and redissolution in deuterated water). The analytical data obtained with these techniques, which were identical to those of authentic EPA and

iPrEPA, are summarized in Table 2. Standard addition of 20 μg/ml of iPrEPA to the water sample and subsequent 300-nl injection followed by matrix removal (−10 kV for 3.0 min) and CE–FPD, further confirmed the presence of the latter compound.

CE–FPD analysis of the aqueous soil extract showed no P-containing peaks in the sample or the blank when applying a 300-nl injection followed by a matrix removal step at −10 kV for 3.4 min. Even after increasing the injection volume to 1000 nl (matrix removal, −15 kV for 6.0 min), no peaks were detected. LODs at this injection volume were previously determined to be in the range of 0.1–0.5 μg/ml [17]. Use of the various MS-based and NMR techniques applied in this study confirmed the absence of alkylphosphonic acids in the soil sample.

The potential of CE–FPD was clearly demonstrated during the Fourth Official OPCW Proficiency Test (1998) when, according to the scenario of the test, a water sample taken at the outlet of a waste water pipe from a chemical facility accused of producing CWAs had to be analysed. A tap water sample taken at the facility was supplied as blank. In this case, the separation capillary had a length of 60 cm. Fig. 4 shows that the 80-nl injection resulted in the appearance of two peaks (t_m 4.68 and 4.89 min) in the sample (trace B), which were absent in the corresponding blank (trace C). Since the peaks were just baseline separated, the compounds were suspected to be closely related. Comparison with a subsequent injection of a reference mixture (trace A) showed the mobility of the unknown analytes to be close to those of EMPA and iPrMPA, which are singly charged with three and four carbon atoms, respectively.

The identification of the two compounds was a challenging task because no authentic compounds were available during the test. Analytical data obtained with the various spectrometric techniques applied are summarized in Table 2. The ^{31}P-NMR spectra revealed that compound **1** possessed a P=O bond as indicated by the observed shift (*d* 33.7 ppm), and compound **2** a P=S bond (*d* 86.5 ppm). As regards compound **1**, ESI-MS–MS of precursor ion m/z 167, which was absent in the blank, showed product ions at m/z 125 (loss of propene), m/z 107 (subsequent loss of water) and m/z 65 (m/z 125, loss of *O*-propyl resulting in H_2PO_2). At this point the

compound could be represented as $C_3H_7P(O)$-$(OH)OC_3H_7$. The propyl–P group was identified as *n*-propyl by MS–MS of the m/z 125 precursor ion, generated in the ion source by applying a relatively high cone voltage (+35 V). The *n*-propyl substituent is characterized by a relatively low tendency for the loss of propene (m/z 83), whereas the isopropyl substituent is typified by low abundance of the loss of water (m/z 107) [21]. Subsequently, GC–EI-MS of the TBDMS derivatives showed that the retention time of the synthesized $CH_3CH_2CH_2$–$P(O)(OH)$-$OCH(CH_3)_2$ differs significantly from that of compound **1** in the sample, giving evidence by exclusion that compound **1** was *n*-propyl *n*-propylphosphonic acid.

Identification of compound **2** as *n*-propyl *n*-propylthiophosphonic acid was performed using the same strategy. Indeed, compounds **1** and **2** are expected to have only slightly different mobilities in CE, with *n*-propyl *n*-propylthiophosphonic acid, most probably because of the higher mass, showing the shortest migration time. Based on the responses of the reference compounds (EMPA and iPrMPA), the concentrations of both compounds were estimated to be about 10 μg/ml.

3.2. Gradient elution μLC–FPD of water and soil samples

During the Sixth Official OPCW Proficiency Test
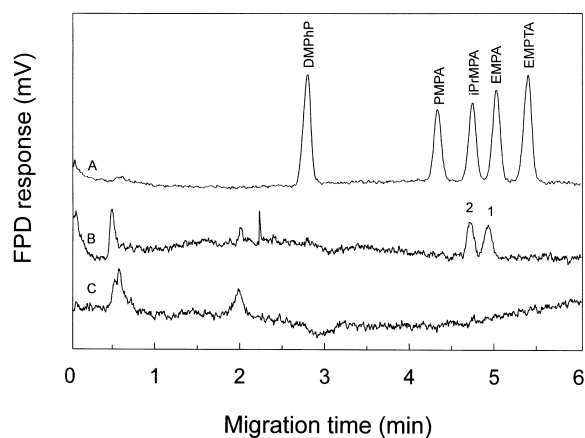

Fig. 4. CE–FPD (P-mode) of (A) a reference solution (20 μg/ml); (B) water sample; (C) blank. Signal of trace A threefold reduced. Injection volume, 80 nl. Same conditions as in Fig. 3.

Table 2
Analytical data of the ESI-MS–MS, GC–MS and NMR methods used to identify the compounds of interest

Compound	OPCW test	Sample	NMR			ESI-MS–MS		GC–MS		Authentic compound available
			Nucleus	Chemical shift (ppm)	Coupling constant (Hz)[a]	Precursor (m/z)	Product (m/z)	EI (m/z)	Derivatization agent[d]	
Isopropyl ethylphosphonic acid	2	Water	P–OC**H**	4.45	d 8.1, sep 6.2	151 [M–H]$^-$	109, 79	209, 67	TBDMS	Yes
			P–**CH**$_2$–CH$_3$	1.56	d 17.1, q 7.7					
			CH–(**CH**$_3$)$_2$	1.26	d 6.2					
			P–CH$_2$–**CH**$_3$	1.06	d 17–20, t 7.7					
			P	30.2						
Ethylphosphonic acid[c]	2	Water	P–**CH**$_2$–CH$_3$	1.39	d 16.6, q 7.8	111 [M+H]$^+$	93, 65	323, 281	TBDMS	Yes
			P–CH$_2$–**CH**$_3$	1.01	d 17.3, t 7.8					
			P	25.5						
n-Propyl n-propylphosphonic acid	4	Water	P–OC**H**$_2$–	ca. 3.9	q 6–7[b]	167 [M+H]$^+$	125, 107, 65	280 [M]$^+$, 223, 181	TBDMS	No
			P	33.7		125 [M+H–C$_3$H$_6$]$^+$	83, 65			
n-Propyl n-propyl thiophosphonic acid	4	Water	P–OC**H**$_2$–	ca. 3.9	c	183 [M+H]$^+$	141, 123, 107	296 [M]$^+$, 239, 197	TBDMS	No
			P	86.5		141 [M+H–C$_3$H$_6$]$^+$	123, 107	254 [M]$^+$, 239, 213, 197, 179, 137	TMS	
Thiodiglycol	4	Soil	CH$_2$–**CH**$_2$–S–	2.74	t 6.3	123 [M+H]$^+$	105, 87, 61, 45			Yes
			CH$_2$–**CH**$_2$–OH	3.74	t 6.3					

Table 2. Continued

Compound	OPCW test	Sample	NMR			ESI-MS–MS		GC–MS		Authentic compound available
			Nucleus	Chemical shift (ppm)	Coupling constant (Hz)[a]	Precursor (m/z)	Product (m/z)	EI (m/z)	Derivatization agent[d]	
Ethylphosphonic acid[c]	6	Water	P–CH$_2$–CH$_3$	1.74	d 17.7, q 7.7	109 [M–H]$^-$	79, 80, 91	254 [M]$^+$, 239	TMS	Yes
			P–CH$_2$–CH$_3$	1.11	d 20.0, t 7.7					
			P	33.5						
Dimethyl ethylphosphonate	6	Soil	P–OCH$_3$	3.68	d 10.7	139 [M+H]$^+$	125, 107, 79	138 [M]$^+$, 110	Not derivatized	Yes
			P–CH$_2$–CH$_3$	1.91	d 17.9, q 7.6					
			P–CH$_2$–CH$_3$	1.12	d 20.7, t 7.7					
			P	41.7						
Methyl ethylphosphonic acid	6	Soil	P–OCH$_3$	3.68	d 10.7	125 [M+H]$^+$	111, 107, 93, 79	196 [M]$^+$, 181	TMS	Yes
			P–CH$_2$–CH$_3$	1.78	d 17.7, q 7.7	123 [M–H]$^-$	94, 91, 79			
			P–CH$_2$–CH	1.10	d 20.1, t 7.7					
			P	37.1						
1,5-Bis(2-hydroxyethylsulfinyl)pentane	6	Soil	S(O)CH$_2$–CH$_2$–OH	4.01	m	257 [M+H]$^+$	163, 119	385 [M–CH$_3$]$^+$	TMS	Yes
			S(O)CH$_2$–CH$_2$–OH	3.12	d 13.8, d 8.2, d 6.0					
				3.03	d 13.8, t 4.5					
			S(O)CH$_2$–CH$_2$–CH$_2$	2.96	m					
			S(O)CH$_2$–CH$_2$–CH$_2$	1.84	m					
			S(O)CH$_2$–CH$_2$–CH$_2$	1.67	m					

[a] m, multiplet; q, quartet; s, singlet; t, triplet.
[b] Overlapping resonances of matrix.
[c] NMR measurements carried out at different pH values.
[d] Derivatization agents: trimethylsilylated (TMS), *t*-butyldimethylsilylated (TBDMS).

(1999), when, according to the scenario of the test, a small-scale facility accused of producing non-declared chemical warfare agents was inspected, water from a waste water collector and a soil (sand) sample from the floor of an umbrella-roofed storage area were provided as test samples. Corresponding blank samples were also provided: tap water, taken at the facility and soil of a similar type taken close to storage area. The water sample and the aqueous extract of the sand sample were subjected to a gradient-elution μLC–FPD (P-mode) procedure, which was recently developed to enable screening for lower as well as higher alkylphosphonic acids in a single run on a PRP-X100 column [20]. The LODs are in the range of 1–800 ng/ml, which meet the required level of 1 μg/ml of the OPCW tests. The gradient profile was as follows: 0–20.5 min isocratic 0.13 M formic acid; next an 8-min gradient up to 0.3 M ammonium formate–methanol (30:70, v/v) and, finally, an 11.5-min hold. The eluent flow-rate was 8 μl/min, and water at 7 μl/min was used as make-up. The use of a make-up solution was essential to maintain stable introduction of the eluent into the FPD during the gradient run. Fortunately, because the FPD is a mass-sensitive detector, its response is proportional to the mass-flux of an analyte. That is, the use of a make-up flow does not reduce sensitivity.

Water sample (20 μl), the corresponding blank and a reference solution containing eight alkylphosphonic acids were injected into the gradient μLC–FPD system. Fig. 5 shows the chromatograms of the water sample (trace B) and the reference solution (trace A). The two peaks showing up in the sample were absent in the blank trace which is not included in the figure. The second peak was tentatively attributed to EPA on the basis of the retention times which were identical (7.75 min). Compound **1** had a retention time (6.71 min) that was slightly larger than that of MPA (6.58 min). As was demonstrated in an earlier study [22], inorganic PA and monomethyl phosphoric acid are likely candidates.

Subsequent ^{1}H- and ^{31}P-NMR, ESI-MS–MS and GC–EI-MS (after evaporating 1 ml of the sample to dryness and trimethylsilyl (TMS) derivatization) amply confirmed the identification of compound **2** as EPA (Table 2). Compound **1** in the μLC–FPD chromatogram was identified as PA by ESI-MS–MS
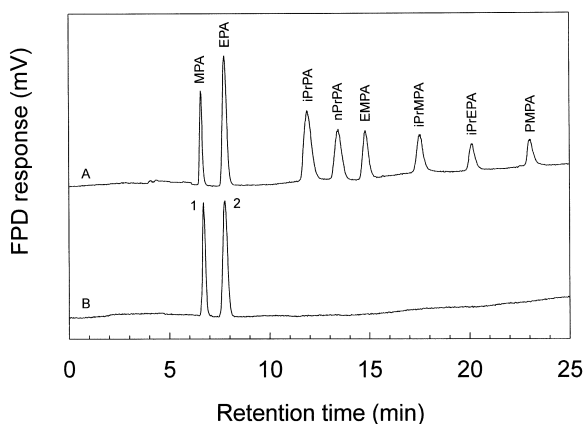

Fig. 5. Gradient elution μLC–FPD (P-mode) of 20-μl injections of a water sample (A) and a reference (B). Gradient programme: 0–20.5 min isocratic 0.13 M formic acid, next an 8-min gradient up to 0.3 M ammonium formate–methanol (30:70, v/v), and finally an 11.5-min hold; flow-rate, 8 μl/min. Make-up, water at 7 μl/min.

(precursor ion m/z 99 [M+H]$^{+}$; product ions m/z 81 and 63 due to the loss of one and two molecules of water, respectively). Because this compound is, for obvious reasons, not included in the Schedules of the CWC, no further identification was carried out.

The sand sample and its corresponding blank were extracted with water according to the procedure described under Experimental. Subsequently, 20 μl of these extracts were analysed by gradient μLC–FPD. Three peaks showed up in the P-mode chromatogram, as is shown in Fig. 6. Compound **1** eluted at 14.37 min, or somewhat earlier than EMPA (14.81 min), which was included in the reference solution (not included in the figure). This compound was identified by NMR, ESI-MS(–MS) and GC–EI-MS (after evaporating 4 ml of a basic methanol extract of the sample and subsequent TMS derivatization) to be methyl ethylphosphonic acid (MEPA) by comparing the obtained data with those of the authentic compound. Relevant analytical data are summarized in Table 2 above. Compound **2**, which eluted after 19.02 min was found to be trimethyl phosphate, which was considered an irrelevant compound in the context of the test. Identification was performed by ESI-MS–MS of the precursor ion m/z 183 [M+H]$^{+}$, which resulted in losses of ethylene giving product ions at m/z 155, 127 and 99 (base peak). Compound
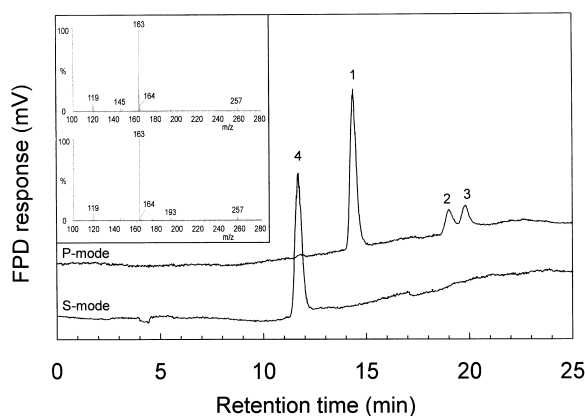
Fig. 6. P-mode and S-mode traces of gradient μLC–FPD of 20-μl injections of an aqueous soil extract. Same conditions as in Fig. 5. Insert: product ion ESI-MS–MS spectra of the m/z 257 precursor ion $[M+H]^+$ of compound **4** (bottom frame) and the authentic compound 1,5-bis(2-hydroxyethylsulfinyl)pentane (top frame).

3 eluted slightly earlier than the reference compound iPrEPA (20.14 min) at 19.83 min, and was identified by the same spectrometric techniques and comparison with the authentic compound to be dimethyl ethylphosphonate (DMEP) (Table 2). Standard addition of the authentic standards of the three P-containing compounds to the aqueous extract of the sample and repeated μLC–FPD confirmed these conclusions.

To screen the soil extract for the presence of compounds related to sulfur mustard or analogues of this vesicant, the same gradient μLC–FPD method was applied in the S-selective mode. The S-mode trace, which has a 10-fold lower sensitivity than the P-mode trace, and is included in Fig. 6, showed a distinct peak at 11.64 min (compound **4**). Flow injection–ESI-MS of the water extract in the positive ion mode gave a molecular ion $[M+H]^+$ of m/z 257. Subsequent ESI-MS–MS of this precursor ion resulted in a product ion at m/z 163 (base peak) which was attributed to $[M+H-HS(O)C_2H_4OH]^+$ and a product ion at m/z 119 due to the subsequent loss of C_2H_4O. This spectrum is similar to MS–MS spectra earlier recorded for 1,5-bis(2-hydroxyethylsulfinyl)-propane (m/z 135 and 91) and 1,5-bis(2-hydroxyethylsulfinyl)butane (m/z 149 and 105) [19]. As can be seen in the insert of Fig. 6, the ESI-MS–MS spectrum of the subsequently synthesized authentic 1,5-bis(2-hydroxyethylsulfinyl)pentane (top frame)

was identical to that of compound **4** (bottom frame). In addition, GC–EI-MS of the TMS residue showed the characteristic $[M–CH_3]^+$ peak at m/z 385 and, again, agreement with the reference spectrum was satisfactory. Finally, ^{1}H-NMR chemical shifts and coupling constants agreed with those obtained for the authentic compound (Table 2), confirming the unambiguous identification. However, the compound actually spiked to the soil sample was 1,5-bis(2-hydroxyethylthio)pentane, a hydrolysis product of the sulfur mustard analogue 1,5-bis(2-chloroethylthio)pentane, which was identified in another test sample, an organic liquid (not to be discussed in this study). The rapid oxidation of closely related hydrolysis products of sulfur mustard analogues, e.g. 1,3-bis(2-hydroxyethylthio)propane and 1,4-bis(2-hydroxyethylthio)butane in soil, in the presence of alcohols, has been reported before [19]. To obtain supplementary evidence for the tentative identification by μLC–FPD discussed above, the aqueous soil sample extract was injected on a column with a different selectivity, viz. a LiChrosorb RP-18 column, with aqueous 25 mM acetate buffer (pH 4.0)–methanol (95:5, v/v) as the eluent. A single peak showed up at 7.08 min, which was the same as the retention time of the 10-μg/ml standard solution of 1,5-bis(2-hydroxyethylsulfinyl)pentane. Standard addition and subsequent μLC–FPD in the S-mode confirmed the identification. The LOD for this compound was 5 μg/ml, or about 100 ng, injected, for both μLC–FPD (S-mode) procedures.

Next to 1,5-bis(2-hydroxyethylsulfinyl)pentane, also 2-(4-pentenylsulfinyl)ethanol, another degradation product of the same spiked parent compound was reported by participating laboratories. No laboratory reported the original spiking compound that was, obviously, converted into the sulfinyl oxidation products in the sand.

3.3. Peak compression μLC–FPD (S-mode) of a soil sample

Another μLC–FPD method, which combines large-volume injection and peak compression, was used to screen samples for the presence of TDG during the Fourth Official Proficiency Test. According to the scenario, a soil sample from a waste pit and a similar type of blank soil close to the pit were

taken. Both were extracted with water according to the procedure described under Experimental. Next, 1% *n*-propanol was added to both extracts to create the peak-compression effect [18]. Subsequently, 10 µl were injected on a LiChrosorb RP-18 column, and eluted with aqueous 50 m*M* ammonium acetate adjusted to pH 4.0 with acetic acid. One prominent S-containing peak showed up in the sample extract above a completely undisturbed baseline, which underscores the selectivity of the procedure. No such peak was present in the extract of the blank soil. From its retention time, the peak could indeed tentatively be attributed to TDG. More importantly, this conclusion could be drawn less than 10 min after extraction of the soil sample had been completed. This is, of course, highly advantageous for the goal in mind, rapid screening. As regards the analytical procedure, the extremely narrow peak shape, i.e. the peak compression, was induced by the high *n*-propanol content of the sample, which effects a local gradient of up to 100% modifier at a specific position in the chromatogram. The on-column concentration was used in order to obtain LODs that meet the present requirements (the LOD was found to be 0.25 µg/ml in our procedure).

Unambiguous identification was based on the closely similar MS and NMR data obtained for the sample extract and authentic TDG. The straightforward results presented in Table 2 do not require additional comment. After standard addition, the TDG concentration was calculated to be 10 µg/ml, which corresponds with at least 20 µg/g TDG in the soil sample.

3.4. Evaluation

Table 2 gives an overview of the results obtained by our laboratory and discussed in this paper, which was focused on the detection and identification of polar (acidic) degradation products in aqueous samples and soils. In most cases presented, a majority or, even, a large majority of all participants could identify the analytes of interest. However, the identification of two compounds, *n*-propyl *n*-propylthiophosphonic acid and 1,5-bis(2-hydroxyethylsulfinyl)-pentane, caused distinct problems. The majority of the participating laboratories apply GC methods, and use GC–MS for unambiguous identification. How-

ever, most of the polar analytes of interest require (sometimes extensive) sample pretreatment as well as derivatization prior to analysis, which may create distinct analytical problems. Our experiences indicate that, if more direct procedures based on LC, LC–MS–MS or CE are applied, these compounds will probably be identified more easily.

4. Conclusions

CE–FPD and µLC–FPD are shown to be very selective and sufficiently sensitive procedures for the screening of CWA breakdown products in water samples and aqueous extracts of soil during OPCW Official Proficiency Tests. Although spectrometric techniques like GC–MS and NMR remain essential for unambiguous identification, liquid-based separation methods such as LC and CE are better suited to deal directly with aqueous samples and polar (acidic) degradation products. This is demonstrated by e.g. the use of (LC–)ESI-MS(–MS) in our laboratory. The several examples discussed above also clearly demonstrate the usefulness of the FPD-based screening procedures which: (i) allow rapid tentative identification, and (ii) provide additional selective (P or S) information on the presence of polar degradation products. The tentative identification in its turn, may give direction to the method of sample pretreatment for GC–MS analysis and NMR, which generally requires a large amount of sample (1–10 ml) to obtain acceptable analyte detectability.

As for the target compounds of the present study, both CE–FPD and (gradient) µLC–FPD were able to detect all spiked compounds, with no false positives being encountered. CE–FPD (P-mode) featured shorter overall time of analysis for screening of the alkylphosphonic acids. On the other hand, µLC–FPD was more robust and sensitive. In addition, µLC–FPD with gradient elution can be used to determine dialkyl alkylphosphonates and their alkyl alkylphosphonic acid degradation products in one run, while the concentration methods necessary to obtain the required sensitivity in CE–FPD are not suitable for the former type of analytes. Sulfur mustard hydrolysis products can be determined by large-volume injection µLC–FPD (S-mode), either

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by gradient elution or isocratic elution combined with peak compression.

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