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Review

Chromatography and mass spectrometry of chemical warfare agents, toxins and related compounds: state of the art and future prospects

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Abstract

Methods for the identification of chemical warfare agents, toxins, bioregulators and related products are frequently reported in literature. These methods are often based on instrumental analysis using chromatography (gas and liquid) and mass spectrometry. Here, these instrumental techniques are discussed in several applications, new developments and trends based on a review of the literature published since 1990. Apart from new instrumental developments, it is shown that modern analytical chemistry can be successfully applied to perform identification in the broad field of analytes ranging from chemical to biological warfare agents. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Mass spectrometry; Chemical warfare agents; Toxins

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1. Introduction

In recent years there has been a growing interest in the analysis of chemical warfare agents (CWAs), as can be derived from the numerous papers in the literature. One of the reasons for this interest is the Convention on Chemical Weapons (CWC) open for signing since 1993. This treaty came into force on 29 April 1997 and should lead to the destruction of an entire category of weapons of mass destruction. Analysis of CWAs and related compounds may play a key role in verifying the fulfilment of the treaty. In order to achieve adequate verification, reliable and sensitive procedures are necessary, commonly based on versatile analytical instrumental methods such as chromatography, electrophoresis, mass and nuclear magnetic resonance (NMR) spectrometry. Moreover, the technology necessary for the destruction of these chemicals needs sufficient analytical back up in order to observe carefully destruction processes [1] or to study alternative destruction methods and to watch over the safety of people involved, e.g., by means of biomonitoring.

Classical CWAs (see Table 1) can be divided into several different groups, of which the most lethal group are the nerve agents. Their name is based on the major action of these chemicals on the nervous system. Nerve agents react with the enzyme acetylcholinesterase in an irreversible reaction in tissue fluid, permitting accumulation of acetylcholine and continual stimulation of the nervous system. Particularly, one nerve agent, sarin (isopropyl methylphosphonofluoridate), was in the news after its use against the population of the Kurdish village of Birjinni in 1993 and after a terrorist attack in the Tokyo underground system on 20 March 1995. The terrorists employed rather impure sarin and a primitive delivery system, yet it was effective enough to kill 12 people and injure more than 5000 others.

The second group, the vesicants, are used for casualty effects. These agents affect the eyes and lungs and blister the skin. Sulphur mustard [bis(2-chloroethyl)sulphide] was frequently used during the First World War and more recently in the Iran–Iraq War [2].

The third group, incapacitating agents, cause great discomfort or produce physiological (vomiting) and/or mental effects on their victims, preventing exposed personnel from performing their military duties.

In addition to the classical CWAs, there is a relatively large group of toxins and bioregulators. In disarmament and other contexts, they are regarded as possible chemical or biological warfare agents (BWAs). Hence, these agents are sometimes also referred to as midspectrum agents. Toxins can be classified according to their physiological action, such as toxins affecting the nervous system (neurotoxins) and those affecting the stomach and intestines (enterotoxins). They can also be grouped on the basis of size; large toxins are generally proteins, whereas the smaller ones are peptides or rather complicated organic molecules, e.g., paralytic shellfish poisons. Toxins can be extremely poisonous, e.g., the *Botulinum A* toxin, produced by the bacterium *Clostridium botulinum*, is one of the most poisonous substances known to man [minimum lethal dose (human), approximately 1 ng/kg] [3]. This value is far lower than that of the nerve agent VX (*O*-ethyl *S*-2-diisopropylaminoethyl methylphosphonothiolate), LD50 8 μg/kg [4] (s.c. guinea pig). Due to the wide spectrum present in nature, and the possibility of large scale production of "new" toxin-producing micro-organisms by gene manipulation, they may not only be difficult to analyse, but may also cause toxicity symptoms that are difficult to diagnose.

In 1971, the Conference of the Committee on Disarmament (CCD), as the forum was called, completed its work on the Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological and Toxin Weapons [5]. This convention entered into force in 1975. Two toxins, however, i.e., the paralytic shellfish poison saxitoxin and the plant toxin, ricin, isolated from castor beans, are on the scheduled list of the Chemical Weapon Convention [6]. This list consists of three schedules of chemicals. The first covers three classes of nerve agents, vesicants (nitrogen and sulphur mustards and lewisites) together with some key precursors as well as ricin and saxitoxin. The second covers three toxic chemicals and precursors which could be used for the production of schedule one chemicals. The third covers a range of toxic chemicals (e.g., HCN, phosgene), small organophosphorus compounds and other precursors which are manufactured commercially on a large scale.

Table 1
Names and structures of selected chemical warfare related compounds

Common name [CAS number]	*Abbreviation*	*Schedule no.*	*Class*	*Structure*
Sarin [107-44-8]	GB	1.A.1	Nerve agent	
Soman [96-64-0]	GD	1.A.1	Nerve agent	
Cyclohexyl sarin [329-99-7]	GF	1.A.1	Nerve agent	
Tabun [77-81-6]	GA	1.A.2	Nerve agent	
VX [50782-69-9]	VX	1.A.3	Nerve agent	
Diisopropyl fluorophosphate [55-91-4]	DFP	-	Nerve agent	
Methylphosphonic acid [993-13-5]	MPA	2.B.4	Decomposition product (G-agents)	

(cont.)

Table 1. Continued

Common name [CAS number]	*Abbreviation*	*Schedule no.*	*Class*	*Structure*
Ethyl methylphosphonic acid [1832-53-7] —	EMPA	2.B.4	Decomposition product (G-agents and VX)	
Isopropyl methylphosphonic acid [1832-54-8]	IMPA	2.B.4	Decomposition product (GB)	
Pinacolyl methylphosphonic acid [616-52-4]	PMPA	2.B.4	Decomposition product (GD)	
O, S-diethyl methylphosphonothiolate [2511-10-6]	-	2.B.4	Impurity (VX)	
Dimethyl methylphosphonate [756-79-6]	DMMP	2.B.4	Precursor and impurity (G-agents)	
Diisopropyl methylphosphonate [1445-75-6]	DIMP	2.B.4	Impurity (GB)	
Mustard [505-60-2]	HD	1.A.4	Vesicant	
Lewisite I [541-25-3]	L I	1.A.5	Vesicant	

Table 1. Continued

Common name [CAS number]	*Abbreviation*	*Schedule no.*	*Class*	*Structure*
Thiodiglycol [111-48-8]	TDG	2.B.13	Decomposition product (HD)	
Thiodiglycol sulphoxide [3085-45-8]	-	-	Decomposition product (HD)	
3-Quinuclidinyl benzilate [6581-06-2]	BZ	2.A.1	Psychochemical	
Saxitoxin [35523-89-8]	STX	1.A.7	Neurotoxin	

In the past, most analytical work was devoted to the classical CWAs and their polar degradation products, in many cases related to the CWC. A comprehensive review on chromatographic analysis of CWAs was published in 1990 by Witkiewicz et al. [7]. Rautio [8], compiled operating procedures for the analysis of CWAs on the basis of work performed by laboratories participating in five inter-laboratory comparison tests. This work was carried out for the benefit of the CWC and was participated in by over 20 laboratories. Recently, Black and Harrison [4] described spectrometric and chromatographic methods for analysis of CWAs and degradation products in a book on the chemistry of organophosphorus compounds.

Today, as a result of new developments in ana-

lytical chemistry focused on mass spectrometry (MS), the analytical chemist can also handle substances of biological origin such as toxins, peptides and proteins. These developments open an entire new field of work for the analyst who should be able to handle a broad spectrum of substances ranging from classical CWAs, their polar degradation products and metabolites, their adducts with biological compounds, toxins, as well as peptides and proteins and even bacteria and viruses.

This article reports on recent developments and gives a view of possible future trends in this special field of work which, due to its wide spectrum of analytes, is certainly related to many other domains in analytical chemistry and biochemistry. This overview is illustrative for the present state of the art and is not intended to be complete.

2. Gas chromatography

2.1. General

Of today's instrumental separation techniques, gas chromatography (GC) was probably the first to be used on a frequent basis to analyse classical CWAs, e.g., nerve agents, in the laboratory; hence early literature references date back to the 1960s [9]. For many years, GC has proven to be an excellent technique for the analysis of CWAs. Except for lewisite, GC can handle almost all CWAs directly due to their sufficiently high vapour pressure and relative thermal stability.

For screening for the presence of CWAs, the use of Kovàts retention indices is still an important tool in GC. In 1991, Hancock and Peters [10] investigated the use of retention index monitoring of CWAs in environmental samples by thermal desorption GC in conjunction with simultaneous flame ionisation detection (FID) and flame photometric detection (FPD). Kokko [11] studied the effect of variations in GC conditions on the retention index measured relative to *n*-alkane and *n*-alkylbis(trifluoromethyl)phosphine sulphide (M-standard) homologous series. Both studies concluded that retention index monitoring is a reliable method for preliminary identification of CWAs. The retention behaviour of 16 species of CWAs representing all types of chromatographic molecular interactions were studied by Huber et al. [12] using the information theory on five types of GC columns to evaluate the smallest number of columns with different stationary phases necessary for a proposed safe identification of CWAs by GC retention data. The authors found that the optimal choice, considering identification power and effort, is a combination of three columns with the stationary phases methyl silicone, 50% phenylmethyl silicone and methylcyanopropyl silicone. This column combination corresponds to forensic safety and can differentiate a maximum of about one million species. However, despite this claimed result, it is widely accepted that identification in relation to the CWC can only be based on spectrometric e.g., gas chromatography–mass spectrometry (GC–MS) results.

Besides the use of retention indices in combination with different types of GC columns, Kaipainen et al. [13] added the use of different detectors to support identification. Their goal was to determine CWAs in air samples by screening a large number of samples in order to select suspected samples for GC–MS analysis. The GC system used consisted of a thermal desorption cold trap injector and an FID system, a photoionisation detection (PID) system and a thermionic detection (TID) system – sometimes called a nitrogen–phosphorus detection (NPD) system. An OV-1701 fused-silica capillary column was connected to a TID–NPD, and an SE-54 fused-silica capillary column was connected jointly, via an effluent splitter, to an FID and a PID. By using these three different detection systems including P, S, N selectivity, useful additional information is obtained. The use of relative response ratios normalised to aniline gave additional information in case of FID versus PID. Results obtained with the TID–PID and TID–FID were less successful due to problems of reproducing selectivity and sensitivity with TID.

The use of the two stationary phases SE-54 and OV-1701 and *n*-alkanes as index standard with universal detectors and *n*-alkylbis(trifluoromethyl)phosphine sulphide (M-series) compounds as index standard with selective detectors is endorsed in the recommended operation procedures (ROPs) [8]. Attached to this ROP is the most comprehensive list of retention indices measured for CWAs and related compounds. However, the availability of relatively inexpensive benchtop GC–MS instruments has re-

duced the use of retention indices as a method for identification significantly.

2.2. Applications

The use of capillary GC for the analysis of classical CWAs has now become routine, explaining the decreasing number of papers describing GC–FID–TID–NPD–FPD applications. Most of the published applications are covered by the review of Witkiewicz et al. in 1990 [7]. Only the GC analysis of lewisite remains troublesome and derivatisation with a germinal dithiol compound is needed for determination at a low (ng) level [14].

Some of the more recent papers describe the use of GC to optimise solid-phase and supercritical fluid extraction methods for nerve agents from water and soil samples, respectively [15,16]. Another paper describes a GC method to study the metabolism of VX in rat plasma [17]. Recently, a GC–FPD method was reported for the analysis of alkyl methylphosphonic acids, possible metabolites of sarin in urine samples [18]. The method was used for the analysis of a large number of urine samples obtained from victims who were exposed to sarin in the terrorist attack on the Tokyo subway. The sarin metabolites were derivatized by trimethylsilylation prior to GC analysis. GC–FPD proved to be a relatively rapid and sensitive method, enabling to deal with the large numbers of samples obtained during the clinical monitoring of the patients.

However, the majority of today's GC applications are carried out in combination with MS, as will be discussed below.

An important field of application, in which GC still plays a key role, is stereoisomeric separations. Several nerve agents possess a chiral phosphorus atom, resulting in pairs of enantiomers. The stereoisomers of chiral organophosphorus compounds may differ considerably in their biochemical and toxicological characteristics. Furthermore, stereospecificity in elimination pathways for chiral organophosphorus compounds has been observed [19], which may have important consequences for the treatment of intoxication. Separation of the enantiomers is important to obtain insight into their in vivo behaviour.

Several GC methods to resolve the stereoisomers of sarin, soman (pinacolyl methylphosphonofluoridate) and tabun (ethyl *N*,*N*-dimethylphosphoramidocyanidate) have been developed by the TNO Prins Maurits Laboratory (TNO-PML) [20,21]. The separations were based on laboratory-made stationary phases, e.g., Chirasil-Val and a heptafluorobutyryl-camphorate nickel stationary phase, enabling the separation of the four stereoisomers of soman [22]. Smith and Schlager [23] reported the use of commercially available cyclodextrin capillary columns to separate the stereoisomers of soman, sarin, tabun and cyclohexyl methylphosphonofluoridate (GF). Five different derivatised cyclodextrin stationary phases were tested. Two of the γ-cyclodextrins and a dipentyl β-cyclodextrin were able to separate tabun, sarin and GF isomers. The separation of the four stereoisomers of soman was accomplished only on trifluoroacetyl and butyryl γ-cyclodextrin columns.

The separation of the stereoisomers of VX by GC has not been reported in the literature. Attempts to separate the isomers of VX on the above-mentioned cyclodextrin columns by our laboratory were, however, unsuccessful [24]. Ultimately, this separation was accomplished by microcolumn liquid chromatography as will be discussed below.

2.3. New developments/trends

There are several new developments in GC such as high-speed GC, comprehensive GC–GC, large volume sample introduction by on-column injection or programmed temperature vaporising (PTV) solvent split injection and new detection methods. Up to now, most of these developments have not applied to the field of CWAs; however, implementation of one of these methods may be just a matter of time.

High-speed GC is based on the use of special inlet systems with relatively short capillary columns operated at unusually high carrier gas flow-rates [25,26]. With these systems, volatile organic compounds can be analysed in seconds instead of minutes. In comprehensive GC–GC, the secondary GC operates as a detector for the first GC system [27]. The method is basically different from two-dimensional GC, commonly known as heart-cutting. In two-dimensional GC, the secondary instrument is not applied to the entire chromatogram eluting from the inlet GC. In

comprehensive GC–GC, this is accomplished by a two-stage thermal modulator, transferring sample portions as sharp pulses to a fast secondary GC by electrically heating the thermal moderator. The second GC should be fast enough to generate complete chromatograms during the time required for a peak to elute from the primary GC column.

To increase sensitivity in GC, large volume sample introduction has been introduced based on either large volume on-column injections or PTV injection. Large volume on-column injections are based on the work by Grob et al. [28,29]. Rinkema et al. [30] described the use of large volume injections for the determination of organophosphorus pesticides, which are structurally related to nerve agents, in water. Recently, the use of large volume injections for the verification of intact organophosphorus CWAs in water samples was explored [31]. Several parameters controlling the injection of a 200 μl sample volume onto a capillary gas chromatographic system were optimised, such as: the injection solvent, dimensions and polarity of the retention gap, the injection rate and the oven temperature during injection and during solvent evaporation. With the developed procedure, the nerve agents sarin, tabun, soman, DFP (diisopropyl fluorophosphate) and VX were determined in freshly prepared water samples at ppt levels. More research will be needed before this method can be routinely applied.

Another way to increase the sensitivity is to improve the detection system. However, detection systems such as FID, FPD and TID–NPD are in fact being used unaltered (except for the improvements in electronics), as reported in the first papers dealing with CWA analysis up to the present. This may either indicate little or insufficient efforts by instrument manufacturers or, on the contrary, may imply the potential of the original design. PID and electron-capture detection (ECD) have been used to a lesser extent in relation to CWAs. Especially valuable for the detection of phosphorus-containing nerve agents and the sulphur-containing mustard related compounds in complicated matrixes are the use of FPD and TID–NPD [7]. As an interesting alternative to FPD for selective detection, the use of sulphur chemiluminescence detection (SCD) has been introduced [32,33]. Essential advantages as compared to FPD are less quenching of carbon-containing compounds, a linear response, and generally, the system is more sensitive than FPD. On the other hand, the FPD is one of the most rigid GC detectors, comparable with FID in ease of operation. This may be a reason why no papers have yet appeared using sulphur chemiluminescence in connection with CWAs.

In recent years atomic emission detection (AED) has proved very useful in the detection of CWAs. With the AED, several elements can be selectively detected at the ng level. It has been used by several laboratories in international training exercises related to the CWC [34] and as an application for the quantification of trimethylsilyl esters of alkyl methylphosphonic acids in environmental samples [35]. Schoene and co-workers [36,37] used AED in combination with MS to study the chemical derivatisation reactions on arsenic-containing CWAs. Moreover, with AED it is possible to calculate an approximate empirical formula for unknown compounds [38]. Another promising new development is the introduction of pulsed FPD [39], which may be a future competitor to the AED. The pulsed FPD is able to separate in time the emission of carbon species from that of sulphur and phosphorus, leading to enhanced selectivity and sensitivity. One further major feature of importance of pulsed FPD is the use of a dual gate approach, of which an example is shown in Fig. 1. In the inset, the carbon, phosphorus and sulphur emission time dependencies are depicted. By means of positioning gate A, the P-emission can be selected without time overlap with the carbon emission, but with an unavoidable small overlap with the sulphur emission. Gate B is further delayed to collect more sulphur emission and less phosphorus emission. By using a variable gain β so that $A-\beta B$ is zero for sulphur (A and B are the gate responses of gates A and B, respectively), the system enables P/S interheteroatom selectivity by more than a factor of 300. With the pulsed FPD system, low detection limits of 180 fg/s (sulphur), 7 fg/s (phosphorus) and 2 pg/s (nitrogen) are demonstrated.

Another promising development is coupling of GC with ion mobility spectrometry (IMS). Actually, IMS may be considered an old technology, because IMS was already discussed by Karasek [40] in 1974 and named plasma chromatography. Briefly, its principle is based on ionisation of analytes by a radioactive

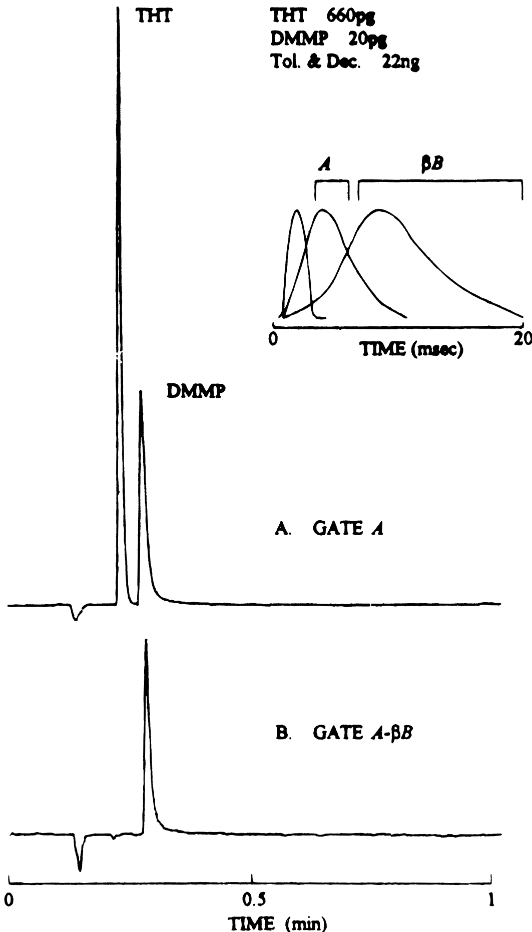


Fig. 1. Dual gate subtraction method to enhance P/S inter-heteroatom selectivity. A 0.22 μl methanol solution of 0.3 m*M* tetrahydrothiophene (THT), 9 μ*M* dimethyl methylphosphonate (DMMP) and 1% toluene and decane was injected using a split ratio of 100:1. A 4-m narrow-bore column was used at 80°C. A GC495 high-pass filter was used [37].

source, e.g., ^{63}Ni (β radiation). Subsequently, the ions are brought into a drift region and move under the influence of an electrical field through a reversed-flow of air or nitrogen until collision occurs with a Faraday cup detector. More information on the basic principles, instrumentation and operation of IMS can be obtained from a paper by Hill et al. [41].

Since the 1980s hand-held devices are available for the detection of CWAs. The combination with capillary GC was accomplished in 1982 [42]. Several improvements have been achieved, especially with the introduction of Fourier transform IMS [43]. Snyder et al. [44] introduced a portable GC–IMS system and investigated the potential of the system for various compound classes including phosphonates and phosphates. Recently, pyrolysis GC–IMS was developed for (bio)aerosol characterisation. Submicrogram quantities of *Bacillus* endospores are detectable based on the presence of picolinic acid and pyridine as biomarkers [45,46]. The availability of field-portable GC–IMS instruments that can provide real-time analytical data is certainly relevant to field-monitoring of CWAs and BWAs.

3. Gas chromatography–mass spectrometry

3.1. General

In the above section it was shown that GC can be an important tool in premonitoring samples for the presence of CWAs and related products. However, for unambiguous identification, the use of MS and more recently tandem mass spectrometry (MS–MS) in combination with suitable ionisation techniques such as electron-impact ionisation (EI) and chemical ionisation (CI) is indispensable. For many years, GC–MS has been used for the identification of classical CWAs such as nerve agents, vesicants and incapacitants, because they are relatively volatile compounds. Compilation of EI mass spectra of CWAs and related compounds are widely available. However, the use of GC–MS has a drawback for the analysis of non-volatile compounds, e.g., related polar degradation products or metabolites. Chemical derivatisation is unavoidable for these compounds and may be complex and time-consuming. Liquid chromatography (LC–MS) methods are, in principle, better suited to deal with these analytes. However, sometimes chemical derivatization is required to record an EI spectrum for unambiguous identification or to obtain sufficient sensitivity performing single ion monitoring. Many applications of GC–MS–MS dealing with CWAs are known and, more recently, of CWA adducts with biological compounds or even of BWAs, as will be discussed below.

3.2. Applications

GC–MS applying EI or CI is routinely being used by all laboratories involved in the analysis of CWAs, as can be derived from the reports on the international laboratory tests organised under the auspices of the CWC. GC–MS applying EI and CI was used by Borrett et al. [47] investigating over 60 alkyl methylphosphonofluoridates in an attempt to find key ions to characterise the whole family of compounds. In another study Sokolowski and Witkiewicz [48] investigated the transformation of sarin dissolved in aliphatic alcohols. D'Agostino and Provost [49] used GC–EI–MS and ammonia CI to study the degradation of tabun in dichloromethane extracts of munitions-grade tabun decontaminated with methanol–potassium hydroxide. Nine components not previously associated with tabun were identified. An unusual study applying GC–MS was based on the viability of using common bivalve molluscs as bioaccumulators of marker compounds in order to verify the production of CWAs in water [50]. The marker compounds *O*-ethyl *S*-ethyl methylphosphonothiolate and diisopropyl methylphosphonate are impurities of VX and sarin, respectively, enabling the presence of VX and sarin in the environment to be marked. The study showed that the clam, *Rangia cuneata*, was able to accumulate and store 25–50-times the ambient concentration of the marker compounds in surrounding water.

The benefit of tandem GC–MS in the field of CWAs was demonstrated in several applications by D'Agostino and co-workers [51–55]. Trace levels of sarin, soman, mustard were detected in spiked extracts of a diesel exhaust (airborne battlefield) environment sampled onto charcoal of a Canadian C2 respirator canister. Due to the complex matrix, GC–EI–MS confirmed trace levels at ng levels [51]. However, lower detection limits in the range of 30–70 pg were obtained using GC–MS–MS reaction monitoring of the collisional activated processes with m/z 158 to 109 or 96 (for mustard) and m/z 99 to 79 (for soman and sarin) [52,53]. GC–MS–MS was further applied to CWA decomposition and hydrolysis products and irritants [54–56].

Fredrikson et al. [57] analysed alkyl methylphosphonic acids in environmental and biological samples using GC–negative-ion CI–MS–MS. An extensive sample preparation was required including derivatisation with pentafluorbenzyl bromide, but an extremely low detection limit (femtograms) of alkyl methylphosphonic acids was reached.

In the 1980s, much attention was paid to the determination of the vesicant sulphur mustard and related compounds in biological samples, soil samples, munition fragments and in the airborne environment. The interest originated from the use of mustard in the Middle East during the armed conflict between Iraq and Iran (1980–1988) [2]. Wide attention was given to the analysis of physiological matrices for confirmation of poisoning in casualties or to understand the metabolic behaviour of mustard for the development of treatment procedures against exposure. In the period 1990–1995, several GC–MS applications on mustard and related compounds were reported [58–66]. Jakubowski et al. [58] studied the determination of thiodiglycol in urine of mustard exposed rats and guinea pigs using GC–MS after derivatisation with heptafluorobutyric anhydride. Wils et al. [59] used GC–MS to analyse mustard and related vesicants in rubber and paint samples in combination with diesel fuel and aromatic white spirit as background. The vesicants were isolated by extraction with methylene chloride or by dynamic headspace analysis. This work was related to the CWC and the samples were artificially prepared. Samples associated with a CWA incident involving sulphur mustard were investigated by Black et al. [61]. The samples were obtained from a Kurdish village in the Northern part of Iraq near the borders of Turkey and Iran and consisted of soil, bomb casing and sheep wool. GC–MS using headspace analysis, solvent extraction and thermal desorption methods successfully confirmed the presence of sulphur mustard and 21 related compounds. In various other papers, Black and co-workers [62–66] described GC–MS methods for the analysis of thiodiglycol and thiodiglycol sulphoxide in urine. Prior to GC–MS, the recovered analytes were derivatised to bis(pentafluorobenzoyl) derivatives. In studies with rats, it was reported that 90% of the administered dose of thiodiglycol was excreted in the 0–24 h urine of which thiodiglycol sulphoxide was the major metabolite [62]. In later papers [65,66], the authors presented the use of GC–MS–MS to detect low levels of mustard metabolites in urine of casual-

ties who had been exposed to sulphur mustard. Fig. 2 shows an example of selected-reaction chromatograms monitoring the fragmentation *m/z* 232→75 of the ammonium adduct of 1,1-sulphonylbis-[2(methylthio)ethane] – the single reaction product from two β-lyase metabolites treated with titanium chloride. The chromatograms for analyte concentrations of 1 ng/ml illustrate the benefits of GC–MS–MS. This demonstrated the possibility of retrospective detection of mustard metabolites in urine samples collected in 1984 and 1988 from casualties of the Iran–Iraq war. A different but promising approach for retrospective detection was recently published by Benschop and co-workers [67,68], who were able to confirm the exposure to sulphur mustard in blood cell samples taken in March 1988 from two Iranian victims. The blood samples were separated into plasma and erythrocytes which were stored at −20°C together with plasma and erythrocyte samples from a Netherlands volunteer which served as a blank. Exposure to sulphur mustard was verified by two independent methods based on either immunochemical analysis of the N7-guanine adduct in DNA and GC–MS analysis of the *N*-terminal valine adduct in globin after Edman degradation and derivatisation by heptafluorobutyric anhydride. Ludlum et al. [69] also used GC–MS as unambiguous proof of sulphur mustard adducts in DNA to support an HPLC method based on fluorescence detection. They reported difficulties in the derivatization procedure

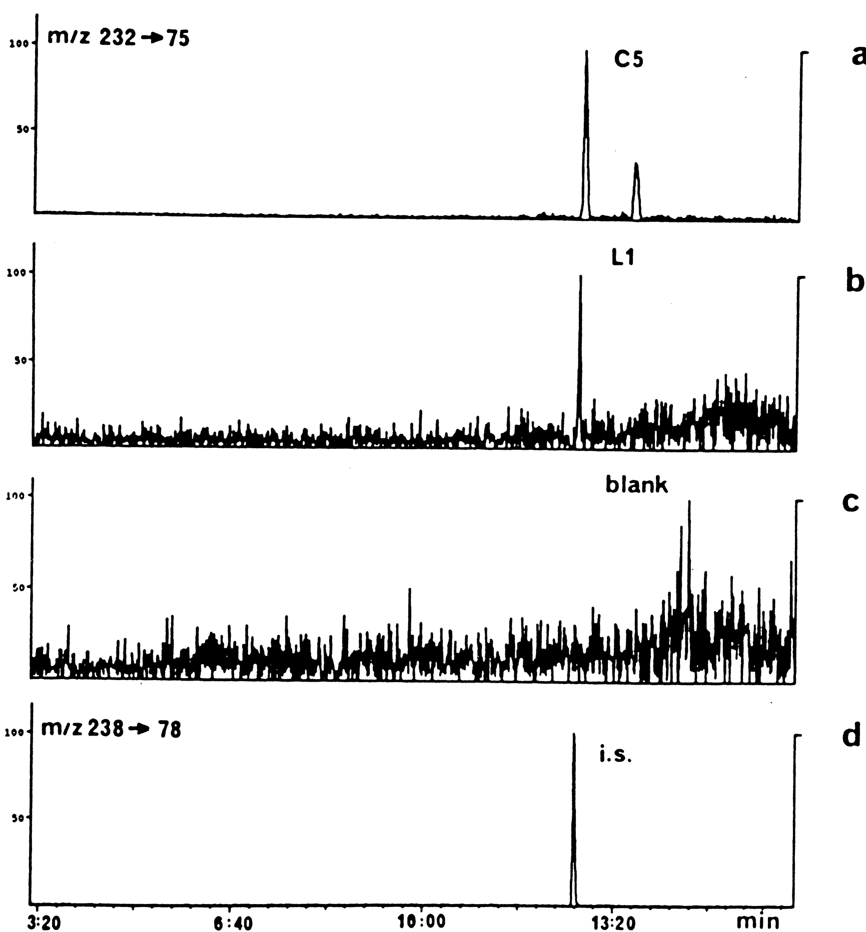


Fig. 2. Selected-reaction chromatograms showing the detection of β-lyase metabolites: (a) Gent patient C5 (1 ng/ml); (b) London patient L1 (0.1 ng/ml); (c) glassware blank for L1; (d) internal standard (5 ng/ml) [62].

necessary to convert DNA adducts to volatile trimethylsilyl esters.

The recent terrorist use of nerve agents in Japan has boosted the development of several GC–MS procedures for biological material. Katagi et al. [70] presented a rapid and sensitive GC–MS method for the identification of EMPA in human serum. The work resulted from a murder case in 1994 in Osaka, Japan. VX was used and its strong killing and wounding properties have caused a great shock. The method presented is based on GC–EI–MS and GC–CI–MS and enabled the unequivocal proof of the use of VX. EMPA was extracted with acetonitrile under acidic conditions from the serum sample, which was previously deproteinized by micro-ultrafiltration. GC/MS analysis was performed after *tert.*-butyldimethylsilyl derivatization with *N*-methyl-*N*-(*tert.*-butyldimethylsilyl) trifluoroacetamide and 1% *tert.*-butyldimethylsilyl chloride. Detection limits of 40 ng/ml and 80 ng/ml were obtained applying full-scan EI and CI mode, respectively.

Recently, two GC–MS applications were published dealing with the Tokyo Subway terrorist attack. Nagao et al. [71] released the sarin degradation products bound to acetylcholinesterase (AChE) in erythrocytes by alkaline phosphatase digestion. The hydrolysis products isopropyl methylphosphonic acid (IMPA) and methylphosphonic acid (MPA) were subjected to trimethylsilyl derivatization and identified by GC–MS. A different approach was carried out by Polhuijs et al. [72]. Their method is based on the high concentration of butyrylcholinesterase (BuChE) in plasma and the fact that BuChE inhibited by the nerve agent sarin can be reactivated by relatively high concentrations (1–100 m*M*) of fluoride ions. The amount of generated sarin in the samples from the Tokyo incident was in the range of 0.2–4.1 ng sarin/ml serum.

3.3. New developments/trends

Developments in GC, such as high-speed GC, large volume sample introduction by on-column injection or PTV solvent split injection, are being successively extended to GC–MS.

The incorporation of quadrupole and ion trap technology into bench-top GC–MS systems introduced MS as a sophisticated GC detector. In several laboratories in the world, these systems prove their value in CWA analysis. Small robust portable GC–MS equipment will be part of the inspection equipment for the verification of CWC.

GC–MS has been developed to a rather mature technique and can be considered as the workhorse in analysis of CWAs. However, the major developments in MS are directed to the ionisation of large biomolecules and in combination of MS with the liquid phase separation techniques.

4. Liquid chromatography

4.1. General

LC as a separation technique with its many modes of operation – reversed-phase, normal-phase, ion-exchange and ion-pair and size-exclusion – is able to separate essentially all compounds that are soluble in a conventional solvent or solvent mixture, with thermolability, polarity and/or volatility not playing a major role. However, its major drawback is the lack of detectors matching selectivity and sensitivity of GC detection systems. This is probably the reason for the many GC applications on CWAs and related compounds even if aqueous sample matrixes are involved and laborious sample treatment or derivatisation is required. The versatility and direct use of LC, however, is seen in the increasing number of LC separations published in combination with MS as will be discussed below. Apart from the combination with MS, LC is expected to increase in importance along with capillary electrophoresis (CE), due to the growing interest in compounds from biological origin, such as toxins, bioregulators and biological adducts of CWAs. Direct use of LC is restricted to compounds that contain chromophoric groups, e.g., incapacitants or compounds which are detectable electrochemically or by conductivity. To compensate for the detection problem, several developments have focused on the detection side.

4.2. Applications

Some recent examples of the direct use of LC are the detection of the polar degradation products and metabolites of sulphur mustard by electrochemical

detection [73], ultraviolet (UV) detection [74,75] and the analysis of ionic nerve agent degradation products by conductivity detection [76]. Clark [73] studied the use of electrochemical detection for the determination of mustard degradation products and amino acid–mustard conjugates. Compounds sensitive to electrochemical oxidation e.g., thiols, alkyl sulphides and alkyl disulphides were detected at 0.5–5 ng levels using a 10 μl injection volume. However, alkyl sulphoxides were detected at higher levels ranging from 30–100 ng. Sulphones, moreover, were not detectable because they are not susceptible to electrochemical oxidation. Comparable detection levels (1–5 ng) were obtained by Raghuveeran co-workers [74,75] for sulphur mustard using UV at 200 nm applying the same injection volume (10 μl). Their method was applied to study the solid-phase extraction of sulphur mustard in blood samples of rats [75]. Kingerly and Allen [76] studied the separation of alkylphosphonic acids on ion-exchange materials and developed a method for analysing soil and natural waters. Detection limits using a conductivity detector ranged from 1–40 μg/l applying 250 μl injections. The above detection methods applied give more or less the same ng detection levels. The lack of sufficient selectivity however, may hamper analysis at lower detection levels in complicated matrixes.

An elegant way to detect intact nerve agents selectively and sensitively was developed by Sipponen [77]. The method was based on post-column enzymatic reaction detection using the enzymes electric eel acetylcholinesterase and human pseudocholinesterase. The system allowed the limited use of organic-containing eluents and was able to detect sarin and soman both at a level of 10 pg and 60 pg for tabun. More recently, Leon-Gonzalez and Townshend [78] applied and improved the work by Sipponen and detected diisopropyl fluorophosphate at a level of 40 pg. It is, however, questionable if these systems will lead to a robust analytical technique.

LC is most suitable for the separation of midspectrum agents including toxins and bioregulators. These compounds are very polar (e.g., shellfish toxins) or may have high-molecular-masses (e.g., biopolymers), excluding the use of GC even after derivatisation. The analysis of shellfish toxins is regularly reviewed in the general referee reports on seafood toxins by Hungerford [79,80]. Analysis of biopolymers has been carried out from the early introduction of LC in the 1970s. They can be sufficiently detected at low (200–210 nm) UV regions and numerous packing materials have been investigated. In the 1996 fundamental review on LC published in Analytical Chemistry [81], it was stated that a plethora of publications for the separation of biopolymers was retrieved during the two-year review period, demonstrating the continuing interest in this area.

4.3. New developments/trends

Current progress in LC is focused on the separation efficiency and, analogous to GC, on the increase of the speed of analysis and the improvement of detection. In the past few years, a wide variety of new stationary phase supports and stationary-phase ligands were added to regular packing materials offering alternative selectivities in reversed-phase, hydrophobic/hydrophilic interaction and ion-exchange modes of LC [81]. Interesting new developments are the use of non-porous small 1–2 μm particles materials and short columns increasing speed of analysis, efficiency and sensitivity [82]. Other promising developments for the separations of biopolymers are the gigaporous highly cross-linked styrenic particles [83], and perfusion supports [84].

With the recognition of the advantages of miniaturised LC [85] starting in the 1980s, new possibilities opened up in the coupling with other systems, e.g., MS [86] and GC detectors [87]. The use of selective GC detection in LC for CWAs and related compounds is advantageous because a number of these compounds of interest contains phosphorus or sulphur atoms. Examples of the use of micro-LC in combination with FPD and TID for CWAs and degradation products have been published by Kientz et al. [88,89]. The interface principle developed is based on the introduction of a jet of liquid plugs or droplets induced by thermal heat, in contrast with other LC interfaces which are based on either nebulisation [90] or evaporation [91]. With the so-called eluent–jet interface, volatile as well as non-volatile compounds can be handled in combination with organic and/or aqueous eluents. The

eluent–jet interface was also applied in combination with EI–MS, as will be discussed below. With the micro-LC–FPD system it was possible to detect simultaneously the nerve agent sarin and its major acidic degradation product IMPA in river Rhine water samples spiked with 10 ppm. The detection limits of the method are in the range 1–20 ppb for lower alkylphosphonic acids (alkyl: C_1–C_5).

A more recent application is the chiral separation of the nerve agent VX [92]. As discussed in the above GC section on chiral separations, the use of GC enables the separation of the stereoisomers of the nerve agents sarin, tabun and soman. However, the separation of the stereoisomers of VX by GC was not possible. Micro-LC proved to be very suitable using a Chiralcel OD column with hexane and 1% isopropanol as eluent and selective GC detection, i.e., TID as shown in Fig. 3. It indicates that selective chiral separation can be obtained at a sufficiently low concentration level of approximately 25 ng/ml.

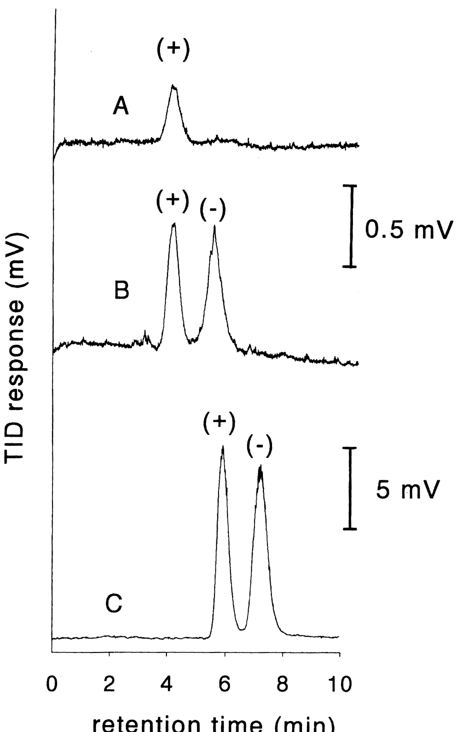


Fig. 3. Chiral separation of VX: column, 250×0.32 mm I.D. packed with Chiralcel OD; eluent, hexane with 1% isopropanol; flow rate, 15 μl/min. (A) 60 nl injection (+)VX (concentration, 30 μg/ml); (B) 60 nl injection of the racemic mixture (concentration of each isomer, 70 μg/ml) and (C) 10 μl of the racemic mixture (concentration of each isomer, 5 μg/ml) [86].

5. Liquid chromatography–mass spectrometry

5.1. Generally

In recent years, LC–MS especially combined with MS–MS has become one of the most rapidly developing analytical techniques. Due to the development of new interfaces and ionisation methods, LC–MS is being extended to an unusually broad field of work including CWAs and related compounds, mid-spectrum agents and recently BWAs. In the 1980s, various types of LC–MS interfaces were available or under development, e.g., moving belt, particle beam, direct introduction, thermospray (TS) and continuous flow fast atom bombardment (FAB) [93]. In relation to CWAs and related compounds, TS-MS was the most successful technique for LC–MS prior to the early 1990s, when the atmospheric pressure ionisation (API) techniques clearly took over. As early as 1988, Wils and Hulst [94] showed the benefits of TS-MS for the analysis of CWA decomposition products shortly after introduction of the commercially available TS-MS developed by Vestal and co-workers [95,96]. However, LC–MS is continuously developing; one of these developments is API–MS, which can handle a broad range of compounds from polar to non-polar across a wide range of flow-rates and mobile phase compositions. It can be divided into electrospray (ES) or pneumatic-assisted electrospray (ion spray) and atmospheric pressure chemical ionisation (APCI–MS). The ion/electrospray as well as continuous flow FAB were developed for the analysis of biological compounds such as nucleotides, peptides and proteins. API–MS allows the analysis of small-medium polar to polar molecules and will most likely replace the use of TS-MS. In combination with collision-induced dissociation (CID), structural information can be obtained and makes this technique an excellent tool for CWA degradation product analysis.

With the growing interest in analysis of biological compounds, e.g., midspectrum agents and biological adducts of CW agents, the use of ion/electrospray

MS–MS increases even for BWAs, as will be shown in Section 5.2.

5.2. Applications

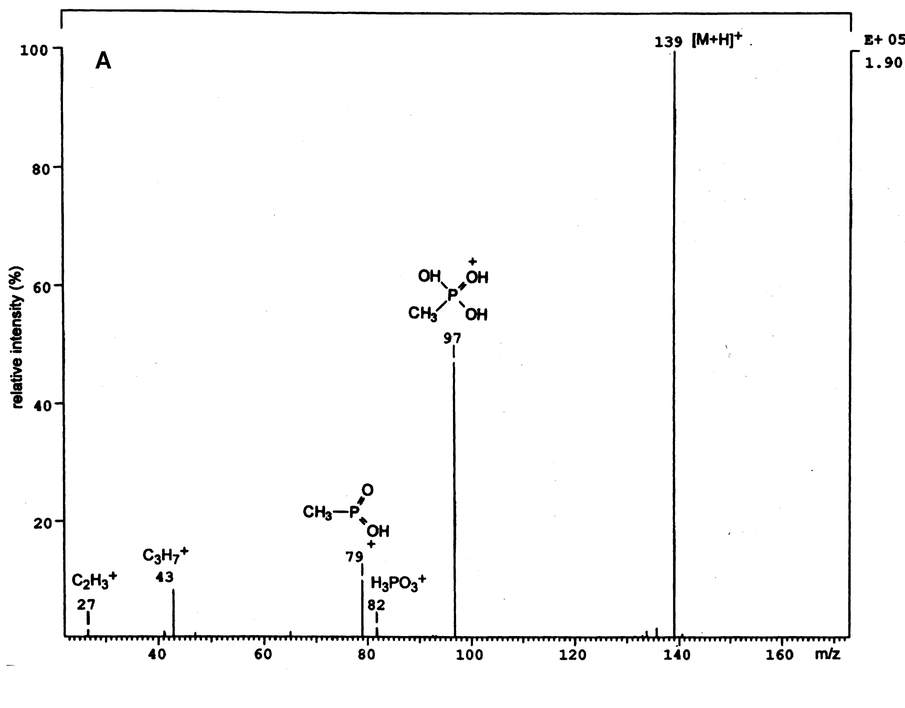
TS-MS was used for the analysis of the nerve agents: VX, sarin, soman, tabun and their degradation products [97,98], the vesicant mustard and degradation products [98,99], mustard adducts [98,100] and saxitoxin [101]. Wils and Hulst [97] demonstrated the differences in the spectra of VX obtained with EI, ammonia CI and TS with an ammonium acetate buffer. They also showed that TS mass spectra depend on the eluent composition. Due to the soft ionisation, TS mass spectra are usually very simple and, consequently, less suitable for identification purposes. Therefore, special techniques have been developed to increase ionisation by means of discharge ionisation, or CID has to be applied in a collision cell of an MS–MS combination. An example of the use of TS-MS–MS was recently presented by Tørnes [102] as a method for the identification of alkyl methylphosphonic acids in aqueous samples. The obtained product-ion spectra gave sufficient information for identification. Moreover, the use of MS–MS also increased the signal-to-noise ratio. Fig. 4 shows an example of product-ion spectra of isopropyl methylphosphonic acid and pinacolyl methylphosphonic acid obtained with TS-MS–MS. Generally, care should be taken with MS–MS identification that identification is based on a sufficient number of ions as well as matching intensity ratios recorded from reference compounds.

Borrett and co-workers [103,104] showed that ES-MS–MS is not only useful for biopolymers. They demonstrated the potential of ES-MS–MS for CWA degradation products in the negative as well as in the positive ionisation mode. Depending on the ionisation mode, protonated and deprotonated molecular ions were observed, including characteristic fragment ions. Black and Read [105] used LC–APCI–MS as a qualitative screening procedure applied to hydrolysis products of nerve agents, sulphur and nitrogen mustards, and 3-quinuclidinyl benzilate (BZ). An acceptable chromatographic separation of 19 hydrolysis products was obtained using a mixed C_8/C_{18}-column and a conventional water–acetonitrile–trifluoroacetic acid (TFA) mobile phase gradient. To screen for the complete range of analytes, the MS was programmed to monitor 14 selected ions in three groups, according to their retention times. CID product ion spectra at a collision cell offset voltage of −25 V (except for 3-quinuclidinol) were used for conformation and identification. The bicyclic protonated 3-quinuclidinol, at m/z 128, gave too little fragmentation for suitable conformation of identification even at an offset voltage of −35 V. The screening procedure was successfully applied to samples collected from the Kurdish village of Birjinni, Iraq (previously analysed using GC–MS and GC–MS–MS [61]) and to samples related to CWC as a part of international collaborative exercises.

Recently, D'Agostino et al. [106] used micro-LC–ES-MS to identify munitions grade sulphur mustard hydrolysis products. The ES-MS data were collected under CID conditions optimised to facilitate acquisition of both molecular and product ion information. The method enabled complete identification of the mustard hydrolysis products, and was also successfully applied to analyse aqueous samples collected at a former mustard destruction site.

Applications still closely related to classical CWAs are studies on biological adducts using ion/electrospray LC–MS. Noort et al. [107] described the use of LC–MS–MS to identify modified sites in human haemoglobin after in vitro exposure to sulphur mustard. Fig. 5 shows clearly that haemoglobin is efficiently alkylated by sulphur mustard; leading to an increase of 104 after hydrolysis. The method is based on cleavage of globin by trypsin and micro-LC–MS analysis of the digests. The alkylated tryptic fragments were assigned upon comparison with a digest from non-exposed globin. The unambiguous assignment of five specific modified tryptic fragments was accomplished by MS–MS [108]. More recently, Noort et al. [109] developed a convenient and rapid micro-LC–ES-MS–MS procedure based on multiple reaction monitoring (MRM) for the quantitative analysis of IMPA, the hydrolysis product of the nerve agent sarin. The method could successfully be applied to detect IMPA at concentration levels of 1–135 ng/ml in serum samples from victims of the Tokyo subway attack and of an incident at Matsumoto, Japan.

The full potential of ES-MS–MS, however, is shown in the biological field, as can be derived from



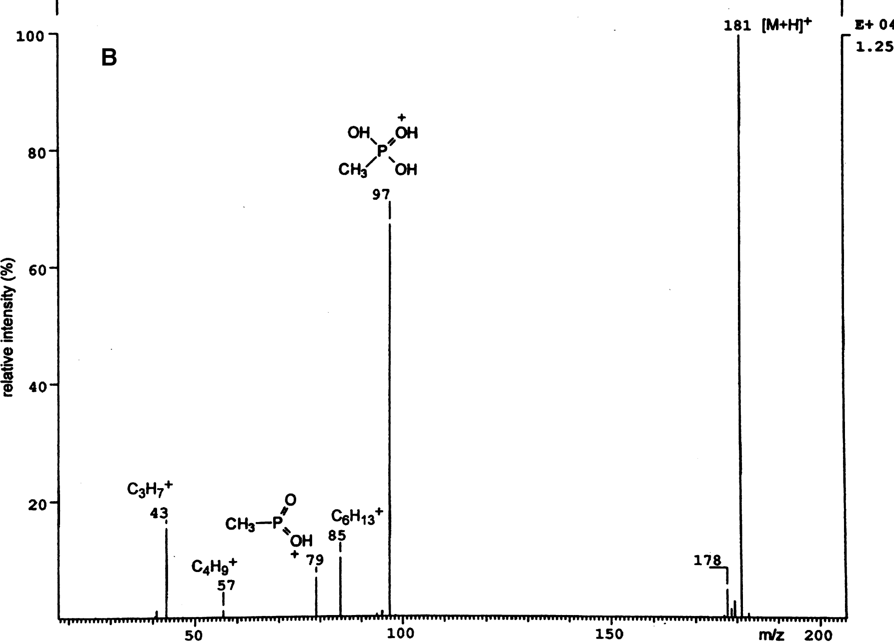


Fig. 4. CID product-ion spectra of the quasi-molecular ion $[M+H]^+$ from flow injection of 100 μg/ml each of isopropyl methylphosphonic acid (A) and pinacolyl methylphosphonic acid (B). Eluent: 0.1 *M* ammonium acetate–methanol (70:30) at 1.2 ml/min. Collision energy: 80 eV. Collision gas pressure (high vacuum): $1 \cdot 10^{-6}$ mbar [96].

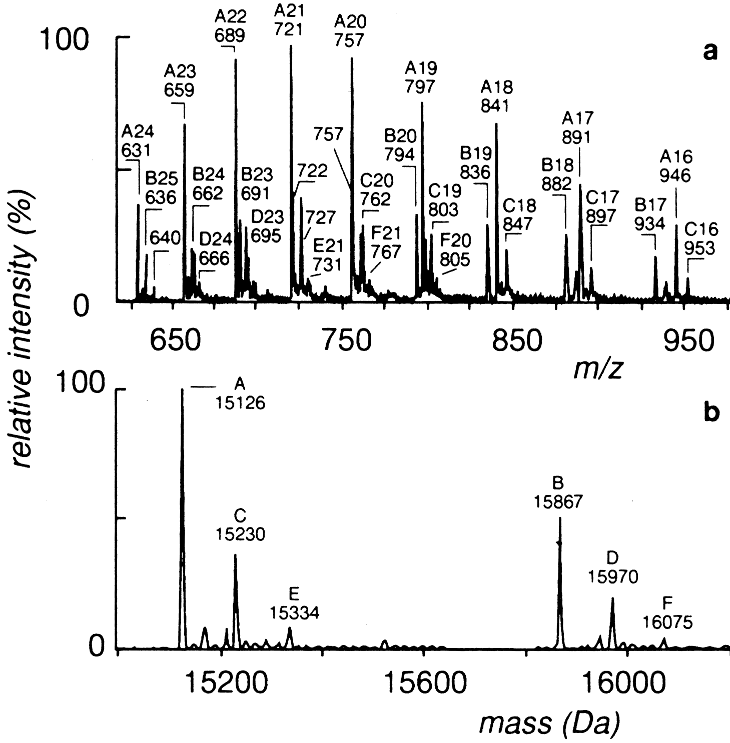


Fig. 5. ES-MS of globin isolated from human blood after exposure to sulphur mustard (25 m*M*). (a) Mass spectrum of multiply charged ions of native α-chain (A) and β-chain (B), monoalkylated α-chain (C) and β-chain (D), and dialkylated α-chain (E) and β-chain (F); the digits denote the number of charges. (b) Reconstructed MaxEnt spectrum obtained by transformation of multiple charged ions to an average mass, represented on a real mass scale; calculated molecular masses for the native α-chain and β-chain of globin are 15 126 (A) and 15 867 (B), and for the monoalkylated species 15 230 (C) and 15 970 (D), respectively [99].

numerous papers. Natural toxins and biopolymers such as plant and fungal toxins, insect poisons, bacterial toxins, reptile and amphibian venoms, and marine toxins and venoms have been investigated in food research, pharmacology, toxicology and biosciences. In recent LC–MS literature, most attention was devoted to marine toxins, e.g., paralytic and diarrhetic shellfish poisons reviewed by Quilliam [110] and cyano bacterial toxins such as microcystins reviewed by Kondo and Harada [111]. Over the last five years, the success of LC–MS in studies of biomolecules even led to an entire new discipline, "biological mass spectrometry" [112]. The potential of tandem MS also increased the use of analyte introduction methods with short columns to separate analytes from interfering ions or flow injections without the use of a separation column.

Krishnamurthy et al. [113] explored the potential of MS and MS–MS techniques for the determination of proteinaceous toxins and antibodies. Flow injections were carried at a flow-rate of 1–2 μl/min into an ES source. In addition to ES-MS, continuous flow frit-FAB and matrix-assisted laser desorption time-of-flight (MALDI-TOF)–MS were used. Molecular masses of a large number of toxins including microcystins, conotoxins, cardiotoxins were recorded with ES-MS and MALDI-TOF–MS. Masses exceeding 100 000 at levels of 0.1–1 pmol were determined. With CID and MS–MS, primary structures of proteinaceous toxins were derived.

Kientz et al. [114] applied (micro) LC–ES-MS for the determination of the high-molecular-mass protein toxin Staphylococcal Enterotoxin B (SEB). With ES-MS, the molecular mass of SEB could be determined accurately at 28 366.3 versus the calculated value of 28 366.1 at a level of 3 pmol/ml. Analysis of tryptic

digests with micro-LC–MS–MS (0.32 mm I.D. column) instead of conventional-size LC–MS–MS (5 mm I.D. column) resulted in a 30–40-fold increase in sensitivity. D'Agostino et al. [115] used (micro) LC–high-resolution-ES-MS using a magnetic sector instrument following on-line LC separation on a 0.53 mm I.D. microcolumn. ES-MS data were acquired for a number of bioactive peptides, including substance P and bradykinins over a wide mass range by scanning the magnetic field sector and calibrating externally with polyethylene glycol standards. The merit of high-resolution ES-CID-MS was demonstrated from results obtained during an international round robin exercise, where the molecular masses of five unknown peptides were to be determined and by the differentiation of lysine from glutamine, differing in residue mass by only 0.0364 u.

5.3. New developments/trends

From an instrumental point of view, focusing on direct liquid introduction methods, API techniques and the use of tandem MS as a whole is a new, major development greatly facilitating the application of LC–MS methods to highly polar molecules and biopolymers and extending the applicability of LC–MS to other fields of work. API techniques have replaced most (if not all) TS techniques and CF-FAB; the clear downward trend of TS-MS in the literature is obvious. On the other hand, different new instrumental developments on liquid introduction and ionisation techniques separate or belonging to API can be expected. An increased use of miniaturised LC techniques in combination with ES interfaces is apparent in the literature. The major reason for using micro- or nanoscale ES-LC–MS is the observed increased sensitivity. In connection with micro-LC and other capillary separation systems, further development in miniaturised ES devices, e.g., nano- and pico-ES, is highly probable. The sensitivity obtained by nano/pico ES will be needed to determine the extremely toxic proteins such as *Botulinum A* toxin at low levels. In addition, modern laser based MS methods in combination with quadrupole, TOF and sector instruments are continuously developing. In addition, Fourier transform (FT) MS in combination with ES and MALDI will provide ultra-high-mass resolution. Further developments of very high-field FT-MS instruments are expected and promising [116].

The smaller polar molecules, such as decomposition products of CWAs or some toxins, can be handled with ES-type interfaces, as demonstrated in the application section. In principle, a part of these compounds can also be analysed on particle beam (PB) MS systems. PB-MS systems have shown great promise, since the EI spectra obtained contain the fragmentation patterns necessary for unambiguous identification. Recently, a new approach was presented based on a jet of droplets (eluent–jet interface) to couple liquid micro-flows directly to the EI-MS system [117]. Liquid introduction by means of a droplet jet is a totally different concept compared to the nebulizer systems used in PB interfaces, i.e., pneumatic nebulizers and thermospray, which all result in a divergent spray of droplets. EI-MS spectra at 1–10 ng levels were found to be comparable with reference spectra and, contrary to many PB systems, linear calibration plots were obtained. Fig. 6 shows an example of the EI mass spectrum of thiodiglycol versus its NIST/EPA/MSDC reference spectrum.

For high-molecular-mass compounds, e.g., mid-spectrum agents or BWAs, the above-mentioned systems based on ES, MALDI and FT-MS will be more extensively used in the future. For BWAs, these systems may even introduce a new and revolutionary method for the determination of intact bacteria and viruses. Despeyroux et al. [118] were able to map the protein content of purified samples of cricket paralysis virus both by direct injection of the purified virus and by on-line LC–ES-MS (Fig. 7). Fig. 8 shows the corresponding electrospray spectra indicating the viability of the technique to detect intact viruses without a preliminary extraction or disruption step. Comparable experiments on pathogenic and non-pathogenic bacteria were carried out by Krishnamurthy et al. [119] using MALDI-TOF–MS. Genus species and strain-specific protein biomarkers were obtained from the measured molecular masses of intact proteins enabling rapid chemotaxonomic classification of micro-organisms.

6. Conclusions

The recent developments reviewed here illustrate the large amount of work and high level of research

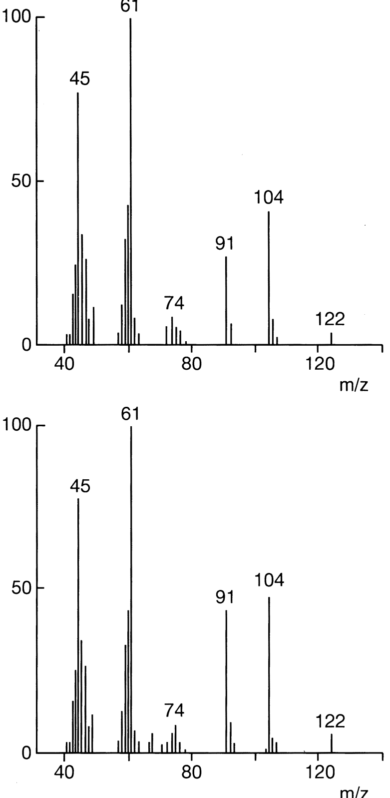


Fig. 6. EI mass spectrum of thiodiglycol (top) obtained with the micro-LC eluent jet interface and a library spectrum (bottom) [107].

carried out to solve analytical problems in the field of CWAs, toxins and related compounds. The instrumental developments and trends which have been discussed are important tools to support activities in the framework of the defence against CW and BW agents. Additionally, recent literature related with the analytical aspects of the CWC shows the advantage of using multiple chromatographic methods to unequivocally identify CWAs, including results from GC–MS (EI and CI), GC–FID, GC–FPD, GC–AED, GC–Fourier transform infrared spectroscopy, as well as LC–MS–MS [120,121].

Concerning GC and (tandem) GC–MS recent developments will probably result in a further reduction of analysis time and detection limits. In the case of CWAs, the use of (tandem) GC–MS with EI and CI, remains probably the most important instrumental method enabling unequivocal identification.

With respect to LC–MS, API techniques are dominant and various new instrumental developments in the area of liquid introduction and ionisation techniques separate or belonging to API can certainly be expected. There are prospects to increase sensitivity after further developments such as FT-MS and miniaturised ES devices, e.g., nano- and pico-ES, combined with miniaturised LC techniques.

The most striking trend since 1990, however, has been the increasing scope of (tandem) LC–MS methods to an extensive scale of analytes ranging from CW to BW agents.

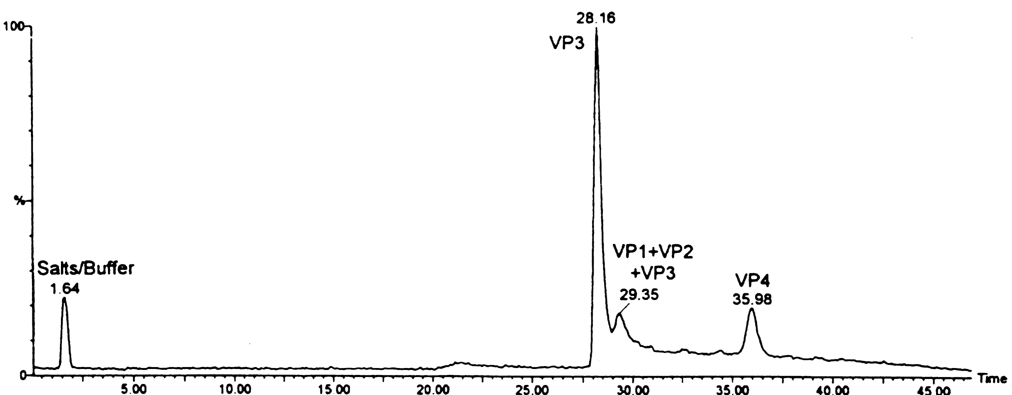


Fig. 7. On-line ES-LC–MS of the purified cricket paralysis virus. Portion of the total ion chromatogram using an Aquapore RP-300 C_8 microbore column. Mobile phase: gradient (30% B to 100% B in 40 min); solvent A: 0.1% TFA–water; solvent B: water–acetonitrile–TFA (10:90:0.1); flow rate, 50 µl/min. Injection: 10 µl of the non-diluted purified viral sample [108].

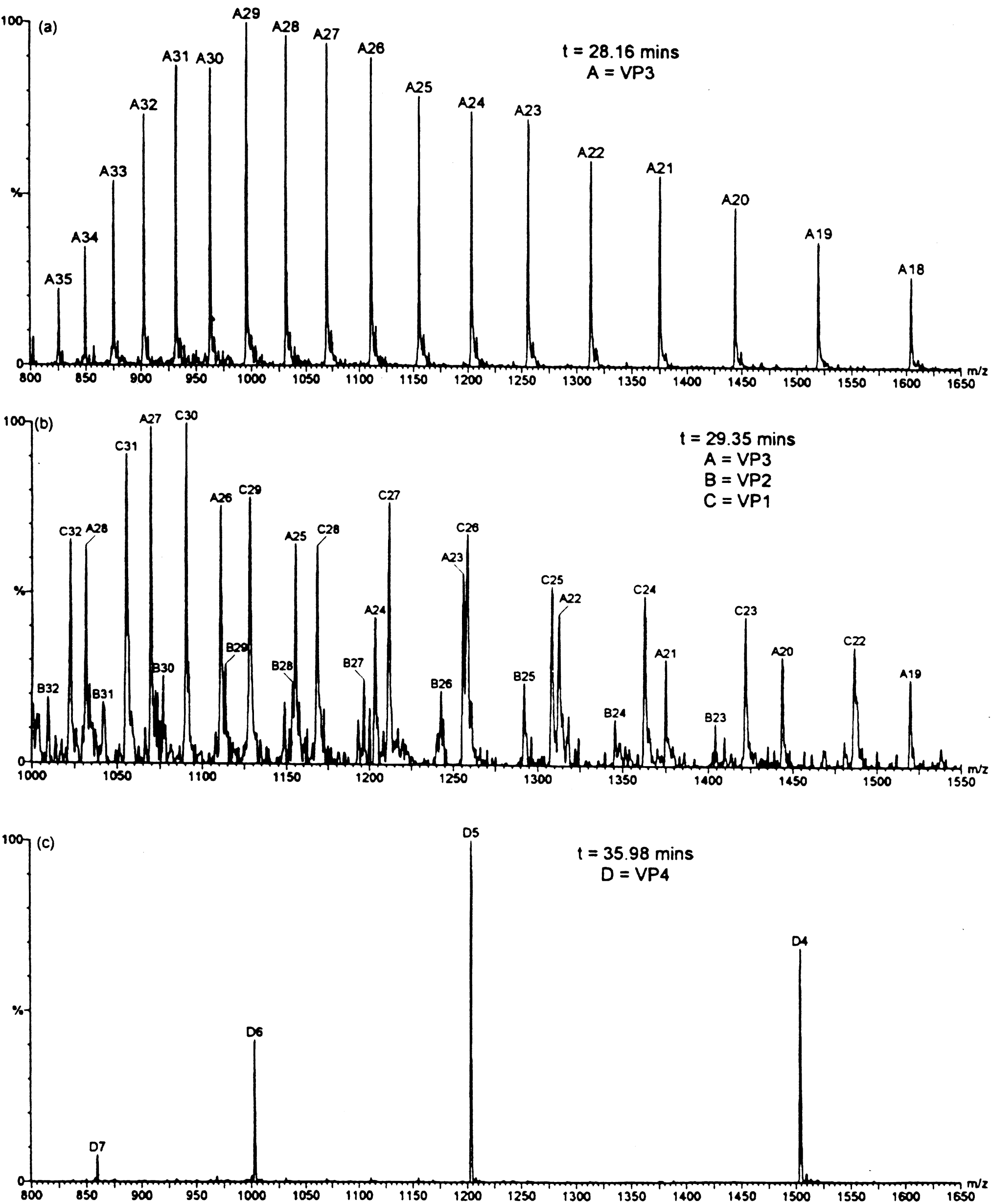


Fig. 8. (a) ES mass spectrum of the first peak to elute, labelled capsid protein VP3 (28 852), retention time t=28.16 min. (b) ES mass spectrum of the second peak to elute, labelled capsid protein VP1 (32 679)+VP2 (32 268)+VP3, retention time t=29.35 min. (c) ES mass spectrum of the third peak to elute, labelled VP4 (6012), retention time t=35.98 min [108].

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