Review of Analytical Methods for the Analysis of Agents Related to Dumped Chemical Weapons for the CHEMSEA project

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1. CHemical Munitions SEarch & Assess (CHEMSEA)

Chemical munitions, though produced in mass during World War II, were never used in battle. Vast magazines of German Chemical Warfare Agents were located in Wolgast, on the Baltic shore. Those munitions, together with others were collected by allies after the war was disposed in the Baltic. Altogether, some 50000 tonnes of chemical weapons were sunk in the Baltic. Official dumpsites were located in the Bornholm Deep, Gotland Deep, Little Belt. Furthermore ships loaded with large quantities of chemical wepons were submerged Skagerrak. Basing on literature sources and occasional shoring /by catches of CWA, the area seem to include also transport routes between Wolgast and Dumpsites, isolated crates/ single munitions spread on the seafloor between Bornholm and the polish coast , and cold war dumpsite in the Gdansk Deep.

In the years 2005-2008 MERCW integrated project has performed mapping and ecological risk assessment of official dumpsites in the Bornholm Deep, Gotland Deep and Skagerrak. The CHEMSEA project aims at filling the gaps in MERCW research (detailed mapping using techniques unavailable in MERCW project, sediment contamination study, corrosion estimation), observing temporal trends in the ecosystem (compare present CWA and degradation products to that observed in MERCW), and extend this research to the largest unofficial dumpsite at the Gdansk Deep.

This report reviews the analytical methods for analysis of chemical agents that are related to the analysis of samples collected by the Chemsea consortium.

1.1. Dumped Chemicals in the Baltic Sea

The use of chemical weapons was extensive during World War I (WW I) aiming to break static lines of defense. However, the World War II (WW II) in Europe was a more mobile war and there was no actual benefit to use chemical weapons against rapid moving units like the German Blitzkrieg. On the other hand, sulfur mustard was used by Japanese and Italy in China and Abyssinia, respectively. The preparedness for using the chemical weapons was extensive leaving large unused storages after the WWII. In the epilogue, according to the Potsdam Agreement, Germany had to be demilitarised and all war material should be distributed to the allies or destroyed. It was also decided that each occupation authority would be responsible for the munitions in is occupation zone. One of the favoured options was the disposal of these munitions by dumping at the sea but to some extent chemical warfare agents (CWA) were also buried or burnt in open pits. If the overall amount which might have been dumped into the Baltic Sea and the North Sea is compared to the amount of captured German CW after WWII, approximately 85 % has been disposed at sea. Different procedures were applied when dumping chemical munitions. The requirement in the agreement was to tow vessels loaded with chemical weapons to the sea was not compiled with. Due to the shortage of ships it is known that chemical weapons were actually thrown overboard in several places and therefore scattered over large areas. The dumped chemicals were sulfur mustard, Arsine oil, Clark I & II, Adamsite, α-chloroacetophenone, phosgene, tabun and nitrogen mustards. Lewisite was not produced by Germany but has also been proposed to be dumped (Söderström et al). The sea-dumping was over many years a major method of choice to get rid of unwanted storage containers or munitions stockpiles. However, increased environmental concern and international conventions, like the “The London Convention” from 1972 that entered into force 1975 further established with “the London Protocol” 1996 the entered into force 2006, gives international regulation of dumping waste into the sea and have more or less stopped dumping at sea the UN resolution on dumping of chemical waste deals with the same question (www.imo.org). Even though, the chemical weapons have been used in a limited number of conflicts, they have been developed and stockpiled until the entry into force of the Chemical Weapons Convention (CWC) on 29th April 1997 that initiated a complete destruction of these weapons. The CWC does not cover material buried at land before 1977 and dumped at sea before 1985 (www.opcw.org).

1.2. Chemical Weapons Convention

The main driving force for the development of analytical procedures for CWA has for the last decades been the Organization for the Prohibition of Chemical Weapons (OPCW) in its work of implementing the CWC. The CWC aims to eliminate all chemical weapons by prohibiting the development, production, stockpiling transfer or use of chemical weapons and each State Party shall destroy chemical weapons or production facilities it owns or possess. Facilities using chemicals that are considered as dual use chemicals are inspected by the OPCW inspection program. For the purpose of implementing the Convention the Verification annex lists toxic chemicals and their precursors, in three schedules, which off-site laboratories designated by the organization should be able to detect and identify. This include Schedule 1; chemicals that has been developed, produced, stockpiled or used as a chemical weapon and with little or no use for purposes not prohibited under the Convention. Schedule 2; a toxic chemical or a precursor to a Schedule 1 or to a chemical listed in schedule 2 and is not produced in large commercial quantities for purposes not prohibited under the Convention or possess a lethal or incapacitating toxicity that could enable it to be used as a chemical weapon. Schedule 3; it has been, or poses a risk to be produced, stockpiled or used as a chemical weapon and that are produced in large commercial quantities for purposes not prohibited under the Convention. Within the OPCW regime there are annual proficiency tests for laboratories that seek to become a designated off-site laboratory required for the verification of the CWC. Dumped chemical weapons like sulfur mustard, nitrogen mustards, lewisite and tabun are still considered to be relevant CWA and they belong to the Schedule 1 chemicals as well phosgene which belongs to Schedule 3 chemicals. The dumped arsenic-containing chemicals Clark and Adamsite are considered obsolete and they are not included into the Scheduled chemicals.

1.3. Screening methods for CWA products

Screening of relevant CWA related compounds aims to identify presences of the active agent or degradation-, hydrolysis- or biological markers that can demonstrated a history of presence and/or exposure of CWA related material. The concern for CBRN-related terrorism has also turned the interest for synthesis related byproducts that can be used in chemical attribution profiles in criminal investigation that can help identifying synthesis route. Furthermore, the CWC states that it is the use of chemicals in warfare that is restricted not only to the scheduled chemicals as well that CBRN related terrorism would probably include more easily accessible toxic chemicals than CWAs. The OPCW regime for analysis of CWA related compounds aims identify chemicals in the environment above ppm in order to be able to fully verify presence of regulated chemicals. However, in environmental and bioanalytical studies are the requirement towards more sensitivity to detect ppb amounts in order to describe a contaminated dumpsite area or exposed organism. In order to detect presence of actual chemicals good sample preparation and screening methods are required. A minimum of sample preparation is preferred prior screening analysis and concern has to be taken so that *e.g.* pH, redox potential, sample drying or sample homogenization does not alter the sample composition in an uncontrolled manner.

Screening methods can be classified as targeted and non-targeted screening. In targeted screening the work procedure are towards listed chemicals whose present should be detected. In CWC -regulated chemicals and other illegal substances it is most often sufficient to obtain a qualitative response. In other areas such as environmental chemistry the qualitative identification also has to be combined with correct quantitative analysis in order to distinguish between naturally occurrence and elevated levels. Typically, this analysis is done using directed analysis with combined gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS) or suitable selective gas chromatographic (GC) detector.

In non-targeted screening, a more general approach is taken to characterize a sample and identify components that are relevant. During this procedure analysis towards selected properties can be of importance. Element selective detectors combined with GC or liquid chromatography (LC) give the information of various heteroatoms such as arsenic, halogens, sulfur and phosphorus. This combined with

well-established analysis with gas chromatography–mass spectrometry using deconvolution routines and library search provide tools for identification of a broad range of compounds. With new improvements in technology using high resolution mass spectrometry combined with LC or GC using exact mass determination to identify unknowns. Non-targeted methods are typically based on full spectra, in which case the sensitivity of the analysis is worse compared to highly sophisticated techniques like selected ion monitoring by using single quadrupole instrument or selected reaction monitoring by using a tandem mass spectrometer.

Extraction methods

The extraction of samples related to CWAs has traditionally been performed with classical liquid-liquid extractions. Samples are typically extracted with dichloromethane to recover hydrophobic compounds and solid samples, such as soil samples, are prepared using aqueous extraction for hydrophilic substances. For polar analytes extractions with water or water miscible solvent *e.g.* methanol, acetonitrile or acetone is performed. Within the Recommended Operating Procedures, of the "Finnish Blue book" [1-3], procedures with water extraction of various material, solid objects, concrete and soil are recommended. For the isolation of nitrogen mustards hydrolysis products, the corresponding tertiary amino alcohols, basic extractions with methanol containing 1% triethylamine alternatively with 0.5 M ammonium hydroxide are performed. The latter is preferred if the extractions are followed by LC-analysis. Samples are prepared prior GC-analysis with derivatisations. Dried water extracts are reconstituted in acetonitrile prior silylation or acidic methanol prior methylation with diazomethane in order to be analyzed with LC-MS.

However, before extraction with organic solvent, wet material would preferably be dried before extraction with evaporation or adding of hygroscopic salt (anhydrous sodium sulfate). Freeze drying may be an alternative prior chemical analysis, but native extraction is recommended prior extraction for biotests [4]. Freeze drying would also cause loss of volatile components. In the standard equipment in any laboratory extraction funnels and ultrasound-generating devices, probe or bath, is normally present. Ultrasound assisted extraction may speed up and increase the extraction yield. However, ultrasound assisted extraction of soil sample may cause exposure of new surfaces that can resorb the compound of interest and therefore gentle shaking will be of better use. The extraction of *e.g.* soil and sediments can be partially automated with equipment that increases the speed of extraction by increase solubility and diffusion by heat and/or pressure like accelerated solvent extraction (ASE), supercritical fluid extraction (SFE) and microwave assisted extractions. Of these methods ASE and SFE have been tested for CWA related material but due to focus on qualitative analysis are there a limited number of labs using the methods.

Accelerated Solvent Extraction (ASE), is an automated extraction method for solid and semisolid samples often used for soil extraction prior environmental analysis [5]. The sample is placed in an extraction chamber and extracted with a suitable solvent, aqueous or organic. The extraction is performed at elevated pressure and temperature that will increase solvent strength and rate of diffusion resulting in an efficient extraction of relevant chemicals.

In Supercritical Fluid Extraction (SFE), a solid matrix is extracted with a supercritical fluid as the extracting solvent [6]. The supercritical fluid is created by controlling temperature and pressure for the extraction solvent keeping it at the transition between liquid and gas. Carbon dioxide is the most common supercritical fluid and its properties can be modified by co-solvents such as ethanol or methanol.

Solid phase extractions

Solid phase extraction from liquid to solid phase extraction (SPE) materials like cartridges, solid phase microextraction (SPME) and stir bar sorptive extraction are integrated in more directed extractions of particular compound classes/matrises [7]. In current CWA screening methods, cation-exchange is used to remove cation (*e.g.* Mg^{2+}, Ca^{2+}) that interferes with silylation in the screening for alkylphosphonic acids [8, 9].

A microextraction method for screening of CWA degradation products in water samples combining extraction and silylation with MTBSTFA has been developed for SPME [10, 11]. In this screening method, an SPME fiber is exposed to MTBSTFA vapors in head space prior and after a 15 minutes water extraction for the derivatisation of polar hydrolysis products from sulfur mustards and nerve agents. The samples are

analyzed by GC-MS. The limit of detection in full scan ranged from 1-200 ppb. In the OPCW proficency test normally 1 ppm (mg / g) is as a requirement for the method. The method was further transferred to hollow-fiber technology [10, 12].

Element specific screening with gas chromatographic detectors

Heteroatoms in CWA of interest can be used for element selective screening. Elements of interest from CWA agents and their degradation products are arsenic (Clark, Adamsite, Pfiffikus and Lewisite), phosphorus; (nerve agents), sulfur, nitrogen (mustards) and in active agents the halogens (chlorine (mustards, arsenicals) and fluorine (nerve agents)[13]. Suitable gas chromatographic detectors for elemental screening of CWA related compounds are (pulsed) flame photometric detector, atomic emission detector (AED) and nitrogen-phosphorus detector (NPD). Less suitable for CWA related compounds are the electron capture detector (ECD) used for detecting electron-absorbing components. This is particularly sensitive to *e.g.* halogens, organometallic compounds, nitriles and nitro compounds. Thus, this technique is selective towards a chemical property and is not as selective as the mentioned element specific detectors but could be a good complement if a simultaneously screening of explosives should be done.

Flame Photometric Detector (FPD/PFPD); Gas chromatography in combination with flame photometric detection is a tool for selective analysis of sulfur or phosphorus containing samples. In the detector are the analytes burned in hydrogen flame and element specific light is selected with an optical filter. This allows the detection of sulfur 394nm and phosphorus 526 nm. Sulfur Sensitivity 4-20 pg/sec Phosphorus Sensitivity 60-900 fg/sec.

This technique is further developed to pulsed flame photometric detection by the use of time dependence of photon emission process from analytes combusted in the hydrogen flame. This extended to the elements to nitrogen and arsenic and also improved linearity and equimolar response.

Atomic-Emission Detector (AED); Gas chromatography in combination with atomic-emission detector (GC-AED) is a broad method for detection of most relevant elements *e.g.* arsenic, chlorine, nitrogen, phosphorus and sulfur. In the detector are the analytes introduced into microwave induced plasma and the plasma spectrum is recorded from the relaxing of the atomized and exited elements. There is usually high selectivity between elements and analysis is normally performed against selected groups of elements and with multiple injections a broad picture of the sample composition can be obtained [14].

Nitrogen phosphorus detector (NPD); Gas chromatography with nitrogen phosphorus detector (NPD) uses thermal energy to ionize and analyze the analytes and the combined nitrogen and phosphorus components can be detected.

Element specific screening with Liquid chromatographic detector

In the inductively coupled plasma (ICP) the analyte will be atomized and the analysis can be performed using spectroscopic or mass spectrometric techniques. Preferably, when analysis is performed with mass spectrometry and then screening can be done for a single element or several heteroatoms.

ICP-MS is routinely used in speciation analysis of elements in environmental analysis and there is as well an increasing interest for speciation in biological samples *e.g.* metals bound to proteins.

Screening with mass spectrometry

In mass spectrometry the analyte molecules are ionized and the mass of formed ions are determinated in a mass analysator. The method is normally combined with a high performance separation technique like gas chromatography, capillary electrophoresis or liquid chromatography.

Targeted analysis MS and MS/MS: In targeted screening with GC-MS or LC-MS instruments, single ion monitoring technique is performed towards the ions known to be formed by the chemicals that are studied. With tandem instruments (MS/MS) more specific analysis can be performed with selected reaction monitoring of targeted substances. For diagnostic fragments will parent ion scan reveal the molecular ion (GC) or psedumolecular ion (LC) and thereby increase the generally of the method for a group of substances.

In GC-MS the Electron ionization (EI) technique has been available for some decades and standard conditions informative spectra's have been recorded. Libraries that are independent of operator or instrument manufacture has been built and those are, in combination with deconvolution programs, powerful tools for targeted screening in complex samples. The NIST library works almost as an international standard library with over 200 000 substances. Within the OPCW regime, the AMDIS deconvolution and analysis software is *de facto* standard were thousands compounds can be included in the search routines were the analyte reference spectra and, if available, retention index is used by deconvolution software to verify the identification.

Targeted analysis with LC-HRMS: High resolution mass spectrometry (HRMS) with stable performances that produce mass determination with a correct elemental composition is used as identification for targeted substances. This analysis is performed with modern instruments like *e.g.* LC-QTOF, LC-TOF or LC-orbitrap.

Non targeted LC-HRMS: The targeted high resolution screening using the determination of elemental composition can be further developed to non-targeted screening mainly with addition of suitable software. A study with non-targeted analysis should preferably include a control sample to be compared with a contaminated sample. Dedicated software is then used to identify differencing peaks. Identity of the peek is suggested from software *e.g.* Chemspider (www.chemspider.com). This analysis is performed with modern instruments like *e.g.* LC-QTOF, LC-TOF or LC-orbitrap.

1.4. Biological marker screening

The screening of biological material is performed in the same manner as the screening of environmental samples even though the matrixes often become more complex. The biological general activity may also be used to characterize the response from exposed organism generating biomarkers for exposure nonspecific to active agent. The general response to exposure to toxic chemicals has then to be sorted out from agent specific biomarkers.

CWAs can form adducts with macromolecules in biological system which adds requirements for the analysis equipment and methods. Adducts may be characterized with hydrolysis protocols to simplify the analysis of conjugated pool. Further studies are then required to identify the actual conjugation site in the macro molecules *e.g* proteins, DNA. After determination of the conjugation sites targeted analysis protocols could be developed and used for screening of exposure.

2. Arsenic-containing chemicals related to the chemical weapons convention

Within the CWC the arsenic containing Lewisites are listed as CWA in schedule 1. These agents were developed in United States and production started at the end of WWI. However, the war ended prior the compounds was ready to be used in the war. Thereafter several countries have produced and stockpiled Lewisite as Lewisite itself or in mixtures with sulphur mustard. Arsenic trichloride is listed as a precursor in schedule 2. All these four chemicals are present in technical grade Lewisite **Figure 1**.

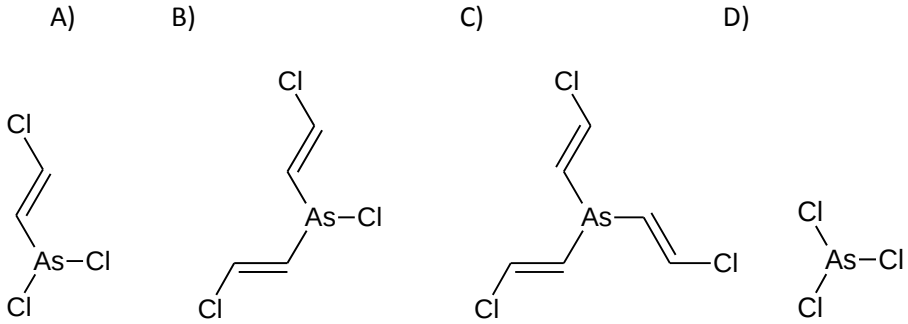


Figure 1. Components of technical (weapons) grade of Lewisite; A) Lewisite I 90%, Lewisite II 10%, Lewisite III less than 1% and Arsenic trichloride less than 1%.

During the development of chemical weapons until WWII several organoarsenic compounds were used as additives in sulfur mustard or as active ingredients in smoke and tear gases The arsine oil and Lewisite were mixed with sulfur mustard in order to enhance the properties of the mustard with lowering freezing point and to create more direct irritation to exposed soldiers',[15]. Furthermore Clark I , Clark II and Adamsite were developed as tear gas or vomiting agents. These types of chemical weapons were developed and produced until the end of WWII and have thereafter been considered obsolete and therefore not included in the CWC **Figure 2-3**.

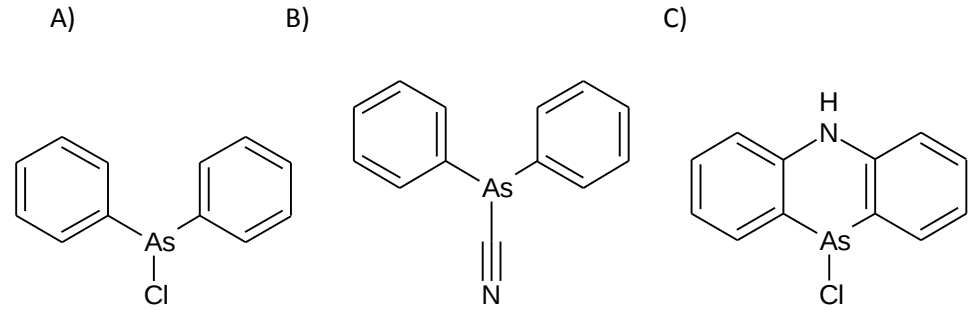


Figure 2 Clark I, Clark II and Adamsite

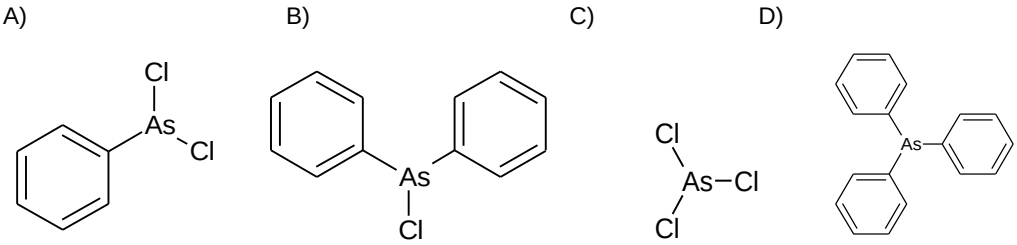


Figure 3 Arsine oil produced by Germany during the WW II contained A) 50% phenyldichloroarsine B) 35% diphenylchloroarsine C) 5% arsenic trichlorid and D) 5% triphenylarsine. [16]

2.1.The environmental fate of organoarsenical agents

Naturally occurring arsenic compounds

Arsenic compounds are naturally occurring in the environment, but elevated levels can also be a result of human activity. Arsenic species that are normally present in the environment can be divided in five different types (Killelea 2003): 1) Inorganic arsenic covalently bound to oxygen or sulfur *e.g.* As_2S_3 and As_2O_3. 2) Inorganic protonated oxyanions *e.g.* arsenic acid ($AsO(OH)_3$) and arsenious acid ($As(OH)_3$). 3) alkylated derivatives of the acids *e.g.* monomethylarsonic acid, dimethylarsinic acid, trimethylarsine oxide 4) arsenosugars from marine organism 5) arsines *e.g.* inorganic arsine and its methylated analogues (AsH_3). In a study by Leivuori and Niemistö (1995), the average measured levels of arsenic in sediment from the Bothnian Bay was 278 mg/kg dw [17]. The average level of arsenic around the dumpsites in Baltic Sea is reported by Paka and Spiridonov 2002 be ca. 25 mg/kg [18].
Arsenic compounds have a tendency to accumulate in biota, and the concentrations of arsenics in fish and shellfish are generally higher than in the surrounding water. The levels of arsenic in food are included in monitoring programs as inorganic arsenics are carcinogenic. For example, the US Environmental Protection Agency (EPA) has set a tolerable daily intake to 0.3 µg/kg/day (WHO 2001).

The most commonly found arsenic compounds in fish and shellfish are the organic molecules arsenobetaine and arsenocholine. In fish more than 95 % of the total arsenic often constitutes of arsenobetaine. It is believed that marine animals are exposed to arsenobetaine through their natural diet, and that the arsenobetaines are produced from inorganic arsenic by algae or other marine organisms. The levels of arsenobetaine in wild marine fish are commonly in the low parts-per-million range. Since arsenobetaine is considered non-toxic, total arsenic is not a good measurement of toxicity.

The weaponised organoarsenic compounds arsine oil, Lewisite and Clark I are chlorinated and the As-Cl bond is hydrolyzed and replaced with an hydroxyl group in contact with water. Analogously is the cyanide group from Clark II hydrolyzed. The hydrolysis of Lewisite is fast and the intact agents cannot be found in water. The hydrolysis is followed by oxidation from As^{III} to As^{V} **Figure 4-5**. In the case of hydrolyzed Lewisite I, chlorovinyl arsonous acid (CVAA) will (as As^{III}) dehydrate to Lewisite oxide (ClCH=CHAs=O) (CVAO) which has low solubility. During the derivatisation with thiol reagent the CVAO is made acidic i.e. converted back to the agent. The CVAO that can be further oxidized (As^{III} to As ^{V}) to 2-chlorovinylarsonic acid (CVAOA) [19]

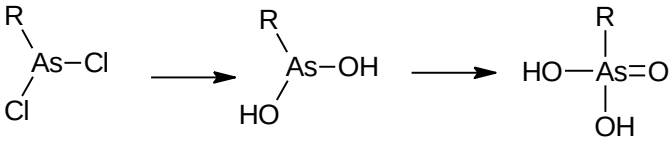


Figure 4 Hydrolysis of dichloro substituted organoarsenial CWA (Lewisite I and phenylarsine)

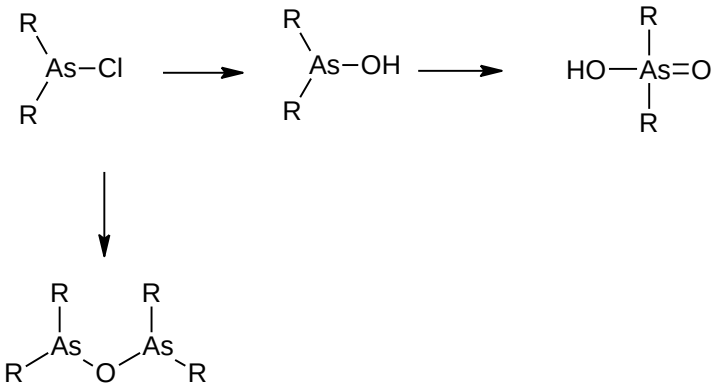


Figure 5 Hydrolysis of monochloro substituted organoarsenial CWA (Lewisite II and Clark I & II). In Clark agents R=phenyl and in Lewisite II R= chlorovinyl.

2.2.The biological fate of organoarsenical agents

Hydrolysis products

Lewisite hydrolysis products have been demonstrated in urine. The hydrolysis product chlorovinylarsonous acid has been isolated from spiked human urine and from exposed guinea pigs [20-23]. These studies were done by GC-MS analysis methods using dithiols, like 1,3-propanthiol, for derivatisation that converts the hydrolysis products of Lewisite I and II to the same derivative by hydrolysis of one chlorovinyl groups of Lewisite II hydrolysis-product. The derivatised analyte was extracted with SPME with a Limit of Detection (LOD) of 7pg/ml urine. Exposure to Lewisite I will generate the hydrolysis product CVAA and the oxidized product CVAOA that can be measured in the urine by LC-ICP-MS. Kinoshita [24] Measured urine from mouse and Stanelle et al [25] analysed metabolites in rat urine after exposure to Lewisite. The LOD were µg/L-level. In the ICP-MS analysis the argon-chloride cluster (m/z 74.93123) formed in the plasma can produce crosstalk with arsenic (m/z 74.92160). Kinoshita avoided this contamination by adding oxygen to form AsO while Stanelle did not find the ArCl signal problematic.

Conjugation

The hydrolysis product of Lewisite I, CVAA, will react with sulfhydryl groups in the living organism. Snider demonstrated this to occur with glutathione, the major endogenous antioxidant produced by the cells, and lipoic acid. Analogously, Chong [26]studied phenyldichlorarsine in erythrocyte and found glutathione as the main binding species in human, pig, hamster, guinea pig, and mouse. In rat the binding was to both glutathione and hemoglobin. The CVAA will coordinate two glutathione molecules. The preference for lipoic acid is because adjacent sulfhydryl groups with two coordinates will favor complex formation with the arsenic **figure 6.** Fidder speculated Chongs results with cysteine bound phenylarsine oxide stabilized by chelating carboxylgroups from adjacent amino acids.

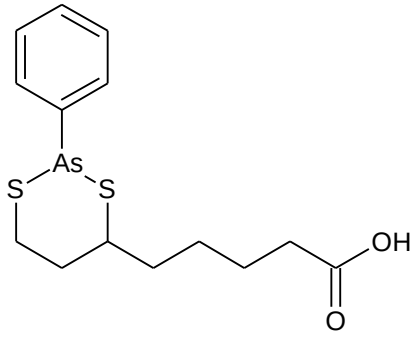


Figure 6 Formed adduct between phenyldichloroarsine and lipoic acid. The lipoic acid is a part of dehydrogenases enzyme complexes for [27]

The fate of Lewisite I in biological system was studied with incubation of radiolabeled Lewisite I *in vitro* with human blood and *in vivo* with guinea pig [28]. The formed radiolabeled products were associated with erythrocytes and 25-50% of the radioactivity was associated with globin. Derivatisation with British anti-Lewisite (BAL; 2,3-dimercaptopropanol) revealed the Lewisite hydrolysis product CVAA. The globin fraction has to be isolated prior derivatisation in order to be separated from the free pool of CVAA since the derivatisation hydrolyzed the adduct and formed the CVAA derivative. The radiolabeled globin was isolated by ultrafiltration with cut off 10 kDa and the globin pool CVAA could be analyzed. The CVAA adduct is potentially formed with two thiol group invicinal cysteines placed in the human and guinea pig globin β-chain. The CVAA in the globin fraction could be detected in guinea pig urine within 12 hours after

exposure and it is extractable from blood for at least ten days after exposure. Total Lewisite and hydrolyzed Lewisite in urine and blood reacted with BAL overnight LOD were estimated to 1nM (blood) 10nM (urine).

2.3.Analytical Methods

The CWA analysis methods used are often based on the methods summarized in the Recommended Operating Procedures presented in "the Finnish blue book" by Verfin [1, 2]. Most chemical weapons agents are volatile and therefore GC-based methods have been chosen for analysis. Typically only the qualitative mass spectrometric data is required for the identification. The combination of electron ionization data containing structural data and chemical ionization data providing molecular weight information are desired. GC-detectors that are element specific *e.g.* NPD-detector for nerve agents, FPD in sulfur-mode for sulfur mustards or AED for various elements including arsenic and sulfur, are in difficult matrixes providing indispensable information about the sample. In the environment, the CWA will start degrading via several processes. These processes include hydrolysis, oxidation, photolysis and microbial activity. Even though, the CWA itself is designed for high toxicity the degradation products are normally non-toxic. Exceptions from this rule are S-2-(diisopropylamino)ethyl O-hydrogen methylphosphonothiolate (EA-2192) formed from VX and Lewisite oxide (CVAO) formed from Lewisite.

Extraction methods

The principle of recovery of the Lewisite's and Clark agents include extraction and derivatisation with thiol-containing reagents prior GC-analysis. The preferred solvents for extraction of an aqueous solution or soil are dichloromethane or hexane with simultaneously derivatisation with thiols in acidified conditions [2, 29, 30].

A comparisonbetween different methods for extraction of CVAA from soil has been performed by Chaudot et al [31]. Soils were extracted with simultaneous derivatisation with 1,2-ethaneditiol using ultrasonic extraction, SFE and ASE. Aging the soil over two months considerably reduced the recoveries of CVAA with 20-75% dependent on soil. SFE and ASE were almost equally efficient, but SFE had more rapid processing time. Ultrasonic extraction clogged the equipment and resulted in low recovery. According to the "Finnish Blue Book" ultrasonic extraction [1, 2] should be avoided and less intense mixing should be done in order to not expose new area into which the analyte may be lost. The SFE and ASE techniques are interesting but has not got any wider usage within CWA analysis probably due to stronger focus on qualitative than to quantitative analysis

If the compositions of the occurring products are of importance the addition ascorbic acid may be added in order to prevent oxidation [32].

Extraction and clean-up of biological samples

Sample preparation prior to extraction with organic solvent, usually includes homogenisation and mixing with pelletized diatomeous earth or hygroscopic salt. The environmentally occurring arsenic compounds are commonly extracted with a polar organic solvent. In a study on accumulation of arsenobetaine in fish after dietary exposure the fish samples (muscle and liver) were freeze-dried and homogenized prior to extraction with methanol-water solution (1:1). The mixture was shaken overnight, centrifuged, evaporated, diluted with water and finally filtrated before analysis [33].

For more hydrophobic analytes, as the phenylarsines, extraction and clean-up methods based on the so called "QuEChERs-method" could be of interest. The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method was developed for extraction of pesticides from fruit and vegetables [34]. A modified method for extraction of fish muscles has been published by Norli et al (2011). In this method the fish sample (5 g) is homogenized and extracted with acetonitrile. Lipids in the acetonitrile phase can be removed by freezing, and further cleaned-up with Primary Secondary Amine. This method has been tested for analysis of a number of organochlorine pesticides, and similar methods have also been used for analysis of organophosphates [35].

Another alternative for extraction of hydrophobic compounds in biota could be to use column extraction with a mixture of acetone and hexane followed by a mixture of hexane and diethyl ether [36]. The drawback

with using nonpolar extraction solvents is that fish lipids will be extracted together with the analytes. However, the amount of lipids can later be reduced with adsorption chromatography or gel permeation chromatography [37].

Derivatisation with thiols prior GC-analysis

The trivalent arsenic forms strong derivatives with thiols and the derivatisation of phenylarsenic-related compounds (arsine oil, Clark) and chlorovinylarsenic (Lewisite) has been extensively investigated[32, 38, 39]. Furthermore, the As^{V} will be reduced by thiols and provide the same products as from As^{III} (**Figure 7**). With thiol derivatisation several benefits will be achieved for the analysis. This solves the memory effect problem on GC-system and prevents deterioration of GC-column. The derivatisation chemistry was originally developed for sulfur selective analysis performed with FPD/PFPD [32]. Later work demonstrated that this derivative is suitable also for GC-AED (As-analysis) and GC-MS [19, 40]. Thiol derivatisation on matrix combined with extraction with organic solvent has been done [29, 32] Properties of the thiol derivatisation procedure alters the composition of the sample. If the status of the individual components is of interest this will be a disadvantage. On the contrary, if the presence of agent is of main interest and sensitivity is problematic the formation of fewer products will be an advantage in trace analysis. The chlorinated and hydrolysed products will generate the same product. When a dithiol is used for derivatisation of compounds with one or two chlorine/hydroxyl groups, the formed derivative will in both cases be the cyclic monosubstituted (phenyl- or chlorovinyl-) product as shown in Figure 7. The most frequently used monothiols are ethanethiol, propanethiol, butane thiol and the methyl ester of thioglycolic acid [39, 41, 42] . The most frequent used dithiols are 1,2-ethanedithiol, 1,3-propanethiol, 3, 4-dimercaptotoluene (DMT) and 2,3-dimercaptopropanol (also known as the antidote British Anti Lewisite; BAL)[28, 39, 41, 43]. Eventhough, Adamsite has been derivatised with propanethiol the formed derivative is not very good for GC-EI/MS analysis (Söderström et al). Two alternative methods have been described. The first method are bromination in boiling glacial acetic acid yielding 2,2´,4,4´,6,6´-hexabromodiphenylamine The second method is in injector conversion into 10-ethyl-5,10-dihydrodiphenylarazine with N,N-dimethylformamide [44].

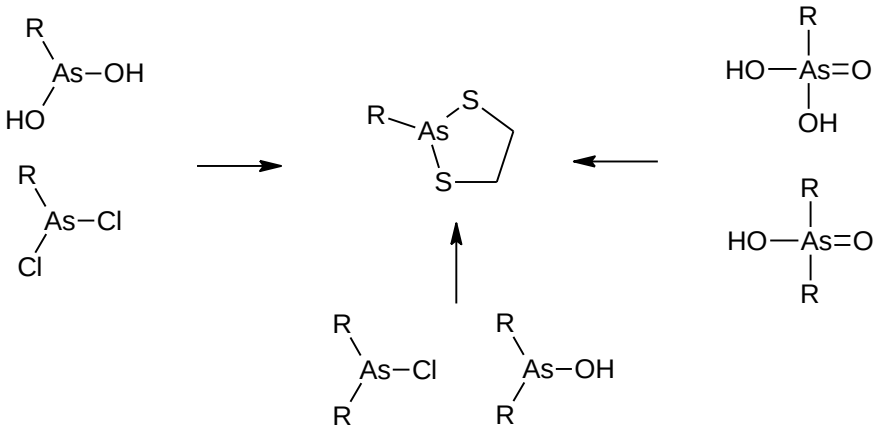


Figure 7 Derivatisation of phenyl- and chlorovinyl-As with dithiols.

Dithiols reduce phenylarsonic acid to phenylarsinoxide. Phenylarsine oxide and phenyl arsine chlorides react with dithiols (1,2-dithiol and 1,3-propanthiol) forming a stable cyclic derivatives thus obtaining a sum of present phenylarsinic compouds [38, 41]. The authors noted that monothiols were not stable in acetone solutions.

The derivatised products are more suitable for partitioning from water to organic phase as compared to their native form. This is an advantage when sample clean-up is required as for example for urine samples. Prior analysis derivatisation was performed with dithiol (1,2-ethanedithiol and 1,3-propanedithiol,

respectively) and the product were isolated by hydrophobic interaction on SPME-fiber and SPE-C18, respectively [20] [22]. Identifications were done by GC-MS and quantification was done by GC-AES Szostek & Aldstadt [45] explored systematic the standard thiol reagents for combined water extraction/derivatisation and recommended the 1,3-dithiol (1,2-dithiol as good but the available reagent had poorer quality) for the extraction out of water and water extracts of soil. They recommended fibers made of poly(dimethylsiloxane) due to robustness prior poly(acrylate)-fiber in spite of higher yield with poly(acrylate) -fiber. The method was suitable for lewisite products but less efficient for phenylarsin products that only were extractable to 10-15%, probably due to low solubility in water[46]. However, in the current methods within the OPCW proficiency test regime propanethiol and butanethiol have become the derivatisations-reagent of choice [2]

An attempt to design a derivatisation procedure for GC-MS analysis of lewisite hydrolysis products was done by Fidder et at[28]. It is known that a fluorine-containing derivatisation reagent will result in enhanced sensitivity in negative chemical ionization GC-MS mode. Therefore the derivatisation was done in two-steps first using British Anti Lewisite for derivatisation of the arsenic species followed by derivatisation of the hydroxyl group of the British Anti Lewisite with heptafluorobuturylimidazol. However, the negative chemical ionization spectrum was dominated by the reagent ion peak from heptafluorobuturyl-moiety. Therefore the authors used GC-MS with electron ionisation instead with a LOD of 40 fmol.

Arsenic analysis with gas chromatography selective detectors

GC-AED: The analysis of the arsenic containing chemical weapons has historically been done with g GC as separation method of choice. Selective arsenic detection may be obtained with in AED. In AED are the column effluent injected into plasma and the plasma spectrum is recorded. This detector allows for immediate determination of the elements present in the analyte solution requiring multiple injections to analyse multiple elements. However, the native Clark and Lewisite and their hydrolyzed counterparts are problematic in a GC system resulting in memory effects and deterioration of the column. Hanaoaka et al (2005) overcome the problem with memory effect by using “on column”-injector when analyzing WWII related teargas munitions called “Red Canisters” containing Clark I and Clark II [40] and abandoned CWA sulfur mustard, Lewisite and Yellow shell (1:1 Sulfur Mustard:Lewisite)[19]. In these reports quantification was done by GC-AED and identification by GC-MS with EI and CI ionization (isobutane). Two types of smoke canisters were studied with pumice and celluloid (nitrocellulose+camphor) as ingredients, respectively. The pumice based canisters contained mostly the dimer bis (diphenyl arsine) oxide (trivalent). While in the celluloid based canisters the next degradation step were dominating diphenylarsinic acid (pentavalent). They have also studied abandoned WWII sulfur mustard, Lewisite and Yellow shell.

GC-FPD: The thiol derivatisation of the organoarsenical CWA was originally done in order to adapt the GC-analysis for FPD detection [32]. With FPD and pulsed-FPD are the GC-column eluate burned in hydrogen flame and the light of the flame is filtered through an optical filter which allows selective detection of sulfur or phosphorus containing compounds. This derivatisation solves also the problem with Clark, Lewisite and their related arsenic hydrolysis products memory effects and deterioration of the GC-column from. Fowler [32] developed derivatisation of CVAA with 1,2 ethanedithiol in order to get selective sulfur analysis in FPD with LOD at 5,5ng/mL. Air samples were collected with impinger and derivatisation with dithiol was performed in the acidified water solution. Ascorbic acid was used to neutralize oxidative species and prevent oxidation of As (100mg/5ml) during the sampling.

GC-MS: The desired method for identification is gas chromatography combined with mass spectrometry GC-MS. The electron ionisation (EI) may be used for identification with the classical spectra interpretation combined with assistance of software support such as deconvolution program *e.g* AMDIS and spectra libraries *e.g* NIST. The chemical ionization technique provides molecular information and if suitable elements are present in the molecule, *e.g* fluorine, sensitive quantification based on electron capture can be performed. Several authors has combined the identification with mass spectrometry with the use of AED or FPD for quantification based on elemental content (As and S, respectively)[40, 46, 47]. The quantification with GC-MS is normally performed in SIM-mode in order to improve the LOD. For quantification two ions are used for each analyte. Limit of quantification (LOQ)s were determined (Söderström et al) from the studies by VERIFIN using GC–EI/MS in SIM mode: triphenylarsine, Lewisite I & II (propanethiol derivative), phenyldichloroarsine (PrSH), trichloroarsine (PrSH): a LOD of ca. 11 µg/kg of sediment.

Arsenic analysis with liquid chromatography-masspectrometry

Wada performed analysis with LC-MS/MS for the detection of hydrolysis products from yellow agent (mustard and Lewisite) and Red agent (Clark arsenic containing species) and preferred electrospray ionization prior Atmospheric Pressure Chemical Ionization due to better sensitivity and broader detection range and better linearity [48]. The HPLC-separation was performed with a gradient of ammonium acetate buffer pH 8 towards buffer with acetic acid with methanol as organic solvent. The pH gradient was needed for adaption for individual species separation and ionization. The LOD varied from 0.1 µg/ml to 0.5 µg/ml. With the method thiodiglycol were analyzed as a ammonium adduct $[M+NH_4]^+$. The naturally hydrolyzed and oxidized Clark agents were analyzed in positive mode and the hydrolysis products of Lewisite with negative ionization

Oxidation with hydrogen peroxide prior LC-analysis: The observation that As^{III} has very low ionizability as compared to As^{V} in electrospray and atmospheric chemical ionization is used in a two-step method. Initially the samples are analyzed in regard to occurring As^{V} components in LC-ESI/APCI-MS. First the samples are treated with hydrogen peroxide in order to oxidize As^{III} to As^{V} products. The total sum of As^{III} and As^{V} will then be measured in a second step of analysis as As^{V} organoasenical compounds. This type of analysis has been performed on Lewisites, Clark, phenyldichloroarsine and Adamsite and their hydrolysis / oxidation products. However, the hydrolysis product of sulfur mustard, thiodiglycol, requires APCI and since the mustard and arsenic related analysis to CWA agents are often performed together, the APCI ionisation method is preferred. (Oral presentations SIBCRA November 2003 [30]). LOQs from the studies by VERIFIN using LC–APCI/MS in MRM mode (after H_2O_2 oxidation) were fore: Adamsite ca. 4 µg/kg; Clark I & II and phenyldichloroarsine ca. 8 µg/kg; Lewisites ca. 11 µg/kg of sediment. (Note: sums of all degradation products), respectively (Söderström et al)

Derivatisation post column LC-analysis: Another strategy for improved sensitivity in LC-APCI-MS is derivatisation with preferably 2-mercaptopyridine or 2-aminoethanethiol in post column derivatisation [49]. The ionisable amine atom will improve sensitivity of the derivatised As^{III} compound. Attempt with thiol reagents 2,3-dimercapto-1-propanol (BAL, British Anti-Lewisite) did not produce any signals for expected derivatization products.

2.4.Arsenic analysis with LC-ICP-MS

In order to verify arsenic containing compounds inductively coupled plasma mass spectrometry (ICP-MS) preferably combined with liquid chromatography may be used. This technique will provide screening for arsenic species with a response quantitatively proportional to the amount of arsenic. This will give a good quantitative determination of arsenic but no information about structure except for the retention time information. This in contrary to LC APCI/ESI MS that will give compound dependent signal but required structure information for qualitative work. Analysis soil samples spiked with arsenic species (As^{III}, As^{V}, DMAA, CVAA, CVAOA) was done by Bass (Bass 1996 [50]) was done by LC-ICP-MS using reverse phase ion-pairing chromatography with the detection limit of ca. 0.1 ppb arsenic. The instrument is linear up to 500 ppb, which would probably be suitable for Lewisite degradation products in sediment samples, but too low for actual arsenic measurements (average blank concentration 25 ppm).

3. Mustard gas

The mustard gases, sulfur and nitrogen mustards, are CWA that causes chemically induced blister on the skin and affects eyes and lungs. The mustards give casualty effects rather than working as directly lethal agents. In its pure form mustard agents is a colorless liquid. Often, in order to improve the physical properties and to obtain more momentary effect sulfur mustard were produced or used in mixtures *e.g.* with arsine oil. The sulfur mustard agents were frequently used during the WWI, by Italy and Japan during the WWII and in the Iran-Iraq war (1980-1988).

Large quantities of mustard agents have been dumped or abandoned in different occasions. Especially, after WWII there were large quantities of unused chemical weapons that needed to be destroyed. A considerable amount of the German stockpile was dumped into the Baltic Sea. The dumped amount was about 25 000 ton sulfur mustard and 2000 ton nitrogen mustards. This method has over the year been the method of choice to get rid of unwanted storage containers or munitions capacity. Even though, the chemical weapons have been used in a limited number of conflicts and they were stockpiled by states. After the ratification of the CWC a process aiming for total destruction of these stocks were initiated.

Degradation pathways of Sulfur Mustards

In the literature the NATO designation of sulfur mustards are often used; H undistilled mustards, HS (used in WWI) and HD (used for distilled mustard 1940 onwards). The main hydrolysis product of sulfur mustard is thiodiglycol. The rate of hydrolysis is decreased in saline solution [51]. In an oxidative environmental will thiodiglycol oxidize to the corresponding thiodiglycol sulfoxide. In the environment thiodiglycol oxidize to the corresponding thiodiglycol sulphoxide, and may also further oxidize to thiodiglycol sulfone in oxidative conditions such as decontamination solutions **Figure 8**.

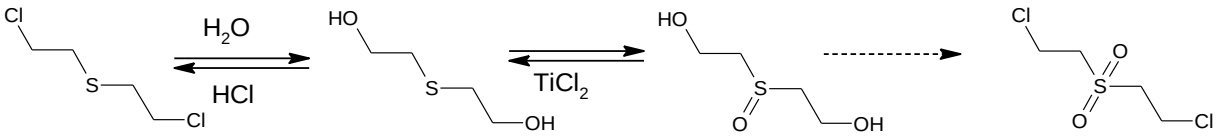


Figure 8. Sulfur mustard hydrolysis and oxidation with assignment of chemical used to reversible the hydrolysis prior GC-MS analysis.

In stored (US) mustard munitions, a solid material called mustard heel has been observed. This material is water soluble and not stable in GC-MS analysis [52]. This product was identified with LC-MS with electrospray ionization as a cyclic sulfonium ion **Figure 9 & 10** [53] Further experiments with mustard destruction demonstrated the cyclic sulfonium ions as possible precursors1 ,4-dithiane and 1,4-oxathiane. These works were performed with direct infusion ESI-MS/MS using methanol as spray-solvent to the water matrix. The same products were also observed on mustard degradation on concrete [54].

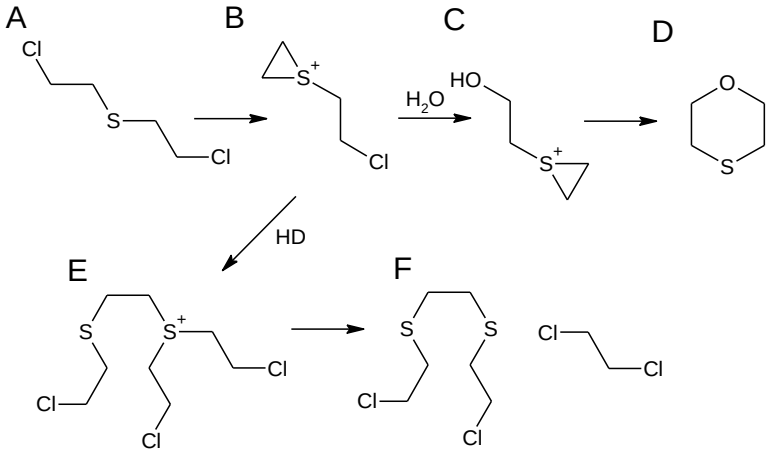


Figure 9 Suggested formation of 1,4-thioxane (D)from sulfur mustard (A) via episulfonium ions (B,C) The formation of a mustard dimer (E) will slowly decompose to sesquiemustard (Agent T) (E) and 1,2dichloroethane. The latter pathway may lead to polymerization of mustard.

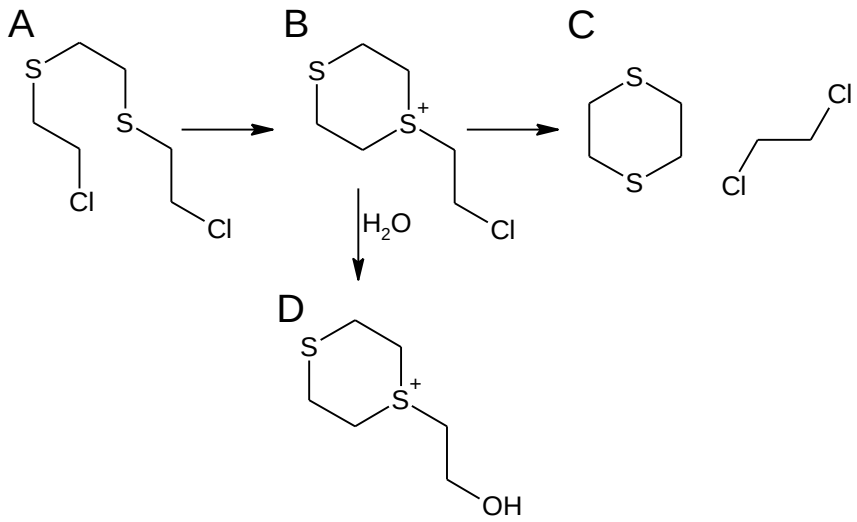


Figure 10. A) Sesquimustard (a mustard synthesis byproduct). B & D) In storage of sulfur mustards a precipitation is noted called mustard heel or tarry. This is a water soluble part that are suggested as the precursors of 1,4-dithiane. C) 1,4-dithiane.

In dumped munitions the hydrophobic sulfur mustards will react on the surface in contact with water which leads polymerization. This means that sulfur mustard encapsulates itself. These lumps are occasionally caught by fishermen during trawling and remains of active mustard are an occupational hazard to be considered. Analyses of the lumps have identified active mustard after fifty years on the sea floor. Analysis of lumps that were caught in a trawl with a combination of GC-AED and GC-MS reveled information of byproducts form the synthesis, *e.g.* sesquiyperite, and the additive Clark I [55]. This type of information is helpful when answering questions of the mustards origin[19].

Analysis of mustard gas and potential degradation products from sediments taken at dumpsite taken in Skagerrak and analyzed with head space trap analysis demonstrated a straight forward method for the analysis of released sulfur mustard and the degradation products especially 1,4-thioxane, 1,3-dithiolane and 1,4-dithiane with LOQ 0.2-0.4 ng/ml **Figure 11** [56].

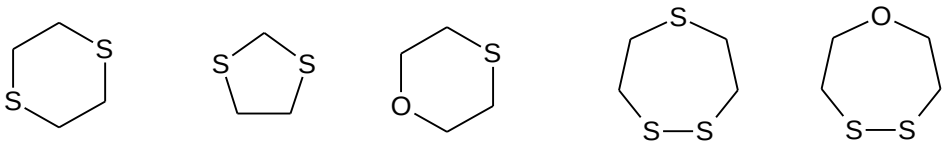


Figure 11 Mustard gas metabolites 1,4-dithiane, 1,3-dithiolane and 1,4 thioxane, 1,2,5-trithiephane and 1,4,5 oxadithiephane identified by Roen et al 2010.

Degradation pathways of Nitrogen Mustards.

Nitrogen mustards have been studied to much lesser extent than sulfur mustards. However, nitrogen mustards could be regarded to behave analogously to sulfur mustard. The corresponding ethanolamine is not considered diagnostic to CWA release due to extensive use in society consumer products and

industrial processes *e.g.* oil refinery. During the WWII, Germany produced 2000 tons that may have been dumped into the Baltic Sea. These agents were also produced by USA. [51].

3.1. Analytical methods for analysis of mustards in environmental samples

Extraction of water samples

Liquid liquid extraction: The standard method is partitioning from water into dichloromethane at neutral to basic (pH 11) conditions. However, this is a compromise for extraction of a broad range of compounds and the polar properties for the expected mustard hydrolysis products, thiodiglycol (sulfur mustard) and aminoalkohols (nitrogen mustards), it may be difficult to obtain good yield. The extraction is improved by using methanol extraction with 1% methanol. With this method the aqueous sample is evaporated to dryness. Thiodiglycol and aminoalkohols are selectively redissolved with 1%trietylamin in methanol and transferred to a new vial and dried. The sample is redissolved in acetonitrile and silylated prior GC-MS analysis [2].

Soil samples are extracted with water and the water fraction are treated as an aqueous sample or alternatively are the soil extracted directly with organic solvent, dichloromethane or 1% TEA in methanol.

Solid phase extraction of water samples: Tomkins tracked thiodiglycol in groundwater near a storage facility in USA. He used solid phase extraction with a sequence of columns with first C18 followed by Amersorb 574 (activated charcoal), The C18 was a guard column and the sample collected on Amberlite were eluted with dichloromethane. The yield was 25-40% and thiodiglycol had a LOD 4-16 µg/L [57]. Liquid liquid extraction in the same system revealed 1% yield with dichloromethane and up to 40% with ethylacetate.

Purification of thiodiglycol and the corresponding sulfoxide in urine were done a combination of Chemelut (diatomeaceous earth) and florisil or C18-columns. Analysis was done with GC-MS after silylation with pentafluorodimetylsilylchlorosilan) (BLACK, CLARKE et al. 1991; Black and Read 1991) The method was improved by using titanium salt ($TiCl_3$) to reduce thiodiglycolsulfoxide to thiodiglycol followed by SPE-extraction on Oasis HLB, a hydrophobic and hydrophilic balanced polymeric material. The derivatisation was improved with heptafluorobuturylimidazole (HFBI). This resulted in much cleaner analysis [58, 59]. Corresponding analysis with diatomeaceous earth column isolation of $TiCl_3$ reduced urine metabolites and analysis of thiodiglycol and β-lyase metabolites after derivatisation with heptafluorobuturylanhydride identification [60].

B-lyase metabolites were purifed from urine with Oasis HLB or ENV+ cartridges. The autors recommended the ENV+ cartridge with DTT added to the water phase and avoiding TFA in order to prevent oxidation of sulfoxides to sulfones[61].

SPME on fiber derivatisation: Sng (1999) at DSO National Laboratories, Singapore has set up a multi-SPME method for the OPCW proficiency test to analyze the degradation products of CWA as their MTBSTFA derivative on GC-MS. The method was tested for mustard degradation products ethyl-2-hydroxyethylsulfide and tiodiglycol and hydrolysis products for nerve gas. SPME fiber is exposed to head space of MTBSTFA before and after extraction in water. The most suitable SPME-fiber for tiodiglycol where PDMS / DVB (65µm) even though Carboxy fiber (75µm) was most suitable as a general SPME-fiber for CWA degradation products. For ethyl-2-hydroxyetylsulfid was the extraction exchange increased 10 times with addition of salt [12, 62].

Head space extraction: Naturally formed degradation products of sulfur mustard may be isolated by headspace extraction. This has been demonstrated sampling with desorption tubes of soil nearby at mustard bomb and sea sediments from dumpsites in Skagerack [56, 63]

Black performed headspace sampling of air from mustard contaminated soil with tenax tubes collecting and compared this with dichloromethane extraction. In headspace at ambient temperature dominated the elimination/hydrolysis 2-(vinylthio)ethanol and the elimination product 2-chloroethyl vinyl sulphide. Changing the soil temperature revealed change in composition of isolated products to 50°C

bis(vinylthioethyl). 100°C 2-methyl-1,3-thioxalane and 2-(vinylthio) ethanol 250°C 1,4-dithiane and 1,4 thioxane. In DCM extraction were thiodiglycol and 2-(vinylthio)ethanol identified and among minor components were mustard gas and the explosives TNT and tetryl identified. The authors recommended the use of thiodiglycol and 2-(vinylthio)ethanol as markers for sulfur mustard contamination during extraction procedures and 1,4-thioxane and 1,4-dithiane during thermal desorption and head space sampling. Analysis was performed with GC-MS (SIM in low ppb).

Analysis of mustard gas and potential degradation products from sediments taken at dumpsite taken in Skagerrak and analyzed with head space trap analysis demonstrated a straight forward method for the analysis of released sulfur mustard and the degradation products especially 1,4-thioxane, 1,3-dithiolane and 1,4-dithiane with LOQ 0.2-0.4ng/ml **Figure 11** [56].

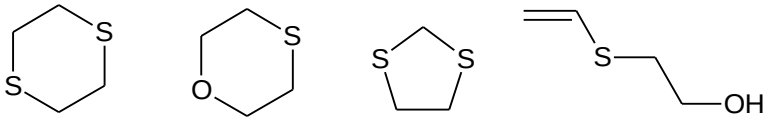


Figure 11 Mustard gas metabolites 1,4-dithiane, 1,4 thioxane, 1,3-dithiolane and 2-(vinylthio)ethanol suitable for analysis for headspace trap analysis

Derivatisation and GC-Analysis

Prior to GC analysis of hydrolysis and oxidation products of mustards derivatisation of the hydroxyl groups are recommended **Figure 12**. In environmental samples thiodiglycol and aminoalcohols can be silylated with BSTFA or MTBSTFA to their trimethyl silyl-or tertbuthyldimethylsilyl esters [2, 23]. The reaction and the formed derivatives are sensitive towards moisture and drying is normally required. The mustard hydrolysis products are volatile and fractions will be lost during evaporation prior derivatisation. The evaporation losses can be reduced by adding HCl to samples to be analysed for amino alcohols (degradation product of nitrogen mustards) and KCl to thiodiglycol samples (degradation products of sulfur mustards) prior to evaporation [64]. The analytes were silylated with MTBSTFA and the analyses were performed on GC-AED for quantification and identification with GC-MS with EI and CI (isobuthane), respectively.

A method with combined derivatisation and extraction with trifluoroacetyl-imidazol of the hydrolysis products of sulfur and nitrogen mustards, thiodiglycol and amino-alcohols has been reported. The sensitivity of the method in GC-MS analysis was in dry soil 5-10µg analyte / gram soil [65].

In biomedical samples is analysis with fluorinated derivatives preferred to be able to use GC-Neg-CI-MS analysis. Most frequent used are the heptafluorobuturyl- or pentafluoro derivatives. Sulfoxides in thiodiglycol sulfoxide and β-lyase metabolites may be reduced with $TiCL_3$ prior derivatisation. Sensitivity after SPE-purification of urine with a LOD 0.2ng/ml[58] 0.04 ng/mL [60] was achieved.

Derivatisation of thiodiglycolsulfoxide may be problematic. Silylation MTBSTFA resulted in four products and therefore BSTFA is recommended. When forming heptafluorobuturyl-derivative, the heptafluorobuturyl imidazole is recommended derivatization reagent and not the corresponding heptafluorobuturyl anhydride, since the released acid induce alteration of the product structure.

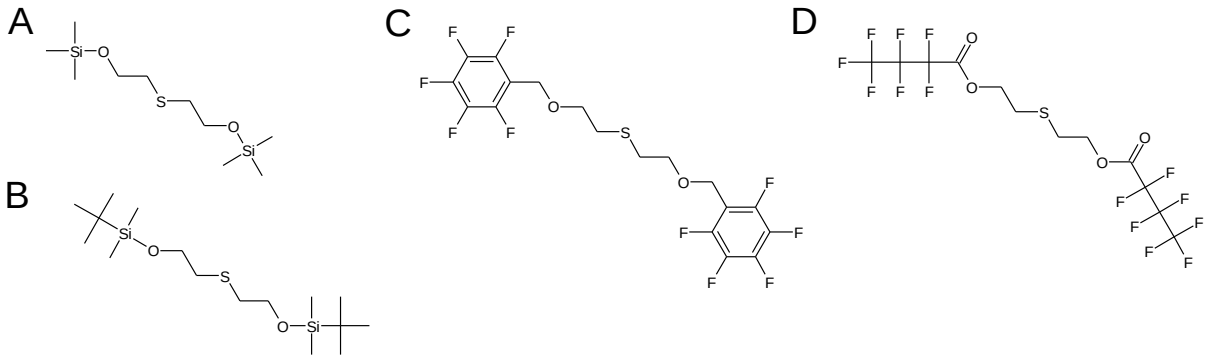


Figure 12. Common derivatives of thiodiglycol for improved GC-properties

Analysis with LC-MS

The interface of LC and MS is today dominated with electrospray (ESI) alternatively atmospheric chemical ionization (APCI). The usage is to large extent overlapping with advantage to ESI with larger and "naturally" ionisable groups while the advantage of APCI is with more stable and neutral molecules. The LC-MS system is in the field of CWA analysis seen as complement to GC-MS in the screening of regulated substances or alternatively used for directed analysis [66].

For analysis of sulfur mustard hydrolysis product thiodiglycol contradictory recommendations are given in literature were APCI is preferred by Söderström et al (Manuscript in preparation) using the psedomolecular ion $[M\text{-}H_3O]^+$as parent ion in LC-MS/MS analysis while Wada et al [48]preferred ESI using a ammonium adduct $[M+NH_4]^+$ as parent ion in LC-MS/MS analysis. The mentioned LOD were 0.01-0.02mg/ml Since mustard in many cases were mixed with arsenic compound oxidation with hydrogen peroxide is used as derivatisation prior LC-MS/MS analysis in order to transfer all arsenic containing compounds from As^{III} to As^{IV} for improved ionization. This procedure will also oxidize thiodiglycol to its corresponding sulfoxide which is suitable for LC-MS/MS analysis with improved sensitivity[30].

Considered to be one of the main advantages with LC-as compared to GC is that the need of derivatisation is avoided. General screening of CWA degradation products including thiodiglcol, thiodiglcol sulfoxide, thiodiglycol sulfone and the amino-alcohols(corresponding to nitrogen mustards) with APCI was evaluated[66]. The analytes were chromatographed on a C8/C18-column alternatively C18-column. The spectra from neutral and basic compounds were dominated by a $[M\text{-}H_2O+H]^+$ pseudomolecular ion and full scan spectra was obtained for 200pg/injection except for the thiodiglycol sulfone 6ng /injections were required. Potential analytes were further studied with LC-MS/MS. The hydrolysis products of mustards, especially sulfur mustads, are studied with APCI which for these products perform more robust results as compared tom ESI [67]. However, ESI has been demonstrated to have corresponding sensitivity with ESI for thiodiglycol and longer chain material *e.g*. agent Q and T [48, 68]. However, the charged dimeric sulfonium species and polymeric material which not soluble in mustard itself (mustard heel) but water soluble will be better analyzed with electrospray [52].

3.2.Biological samples

Analytical method for analysis of mustards in biological samples

Allegation of use of chemical weapon in the Iran-Iraq war was the driving force to develop the analysis of blood and urine samples from victims to establish forensic evidence and the preferred results was retrospective analysis from blood and urine. Therefore, the obtained knowledge is from incubation to human *in vitro*-system or animal in vivo system, *e.g.* used animal system, chosen to assist the understanding of the human system verified with samples from exposed victims here might be possible to

extract some active agents from biological samples. However the metabolism of hydrolysis and oxidation products alternatively acting as alkylating agent towards proteins, peptides or DNA will occur relative rapidly and therefore will there be more relevant to look for hydrolysis products like thiodiglycol and "thiodiglycol adducts" (2-hydroxyethylthioethyl (HETE-) moiety). In humans hydrolysis products will be observed in urine for 24 hours while further metabolized such as adducts to proteins are possible to identify in blood within weeks to months. The hydrolysis-oxidation products are seen in urine mainly as thiodiglycol sulfoxide. The mustard agents are bialkylating agents that act through nonspecific electrophilic alkylation with the episulfonium ion as the active intermediate leaving a HETE-adduct after hydrolysis of the second chlorine. In urine β-lyase products from glutathione-thiodiglycol and N-acetylcyctein products are present. In blood is HETE-adducts to serum albumin and hemoglobin are the key products for retrospective identification.

Hydrolysis products in urine

HCl regeneration of mustard gas from thiodiglycol in urine: The expected hydrolysis product, thiodiglycol, was identified *e.g.* by adding concentrated HCl to the sample (urine) and reversed the product back to mustard gas with head space-sampling with reported LOQ of 10ng/10ml. Problems with background levels of thiodiglycol in controls, up to 55ng/ml made this method not enough reliable as Since false positive occurs [69].

Low molecular mustard gas hydrolysis and oxidation products in urine: Further studies of metabolites in (rat) urine revealed ten products that originated from sulfur mustard. Two metabolic pathways were identified. The first pathway is hydrolysis of mustard to thiodiglycol. The hydrolysis product thiodiglycol oxidizes to corresponding sulfoxide and tom some minor extent to the corresponding sulfone identified with analysis as bis(pentafluorobenzoate)-derivatives in GC-MS [70]. Furthermore a presumed major pathway of formation of a bis-glutathione conjugate is that metabolized and released by enzymatic β-lyase activity excreted in urine as bis-cysteinyl conjugate and its metabolites. These methylated and oxidized β-lyase products demonstrated to be suitable for forensic identification. The metabolism was characterized through feeding with radiolabeled mustard and separate the formed metabolites with HPLC with radioactivity detection and identifying with particle beam LC-MS[71]. These compounds may be analyzed as is with LC-MS and electrospray ionization [61, 72].

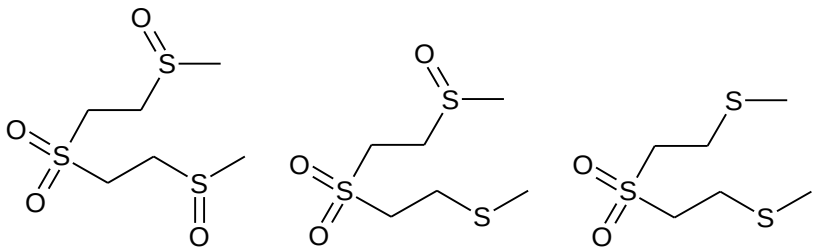


Figure 13 Left and middle β-lyase metabolites and Right the resulting common analyte after reduction with $TiCl_3$.

In GC-analysis are the performance improved if the sulfoxides in thiodiglycol sulfoxide and the β-lyase products are reduces to the corresponding sulphide with titanium trichloride (not reducing the sulfone moiety) **Figure 13.** The analysis is performed after derivatisation to the corresponding bis(pentafluorobenzoate)-derivatives. Preferably, a PTV-injector should be used in the GC-system in order to avoid thermal degradation. The analysis was done by GC-MS and further on by GC-MS/MS improving LOD from 2-5ng/mL urine to 0.5ng/mL urine[73].

A third pathway to track the fate of sulfur mustard is HETE-adducts formed towards DNA. From DNA repair is urinary metabolites of mustard alkylated DNA constituents found such as N7-(2hydroxyethylthioetyl)-guanine. DNA adducts are generally more short-lived as compared protein adducts and GC-MS analysis after necessary derivatisation was found problematic while LC-MS/MS analysis is preferred.

Mustard adducts with proteins in blood

Initial work with feeding radiolabeled mustard gas to blood revealed that most were reacting towards hemoglobin and plasma serum albumin. Hemoglobin has been preferred to study since serum albumin has a higher turnover (7 days as compared to a month) [74]. Identified adducts are towards the amino acids in hemoglobin cysteine, aspartic acid, glutamic acid, histidine [75, 76]. Even though glutathione are expected to be alkylated it was only identified after treatment of hemoglobin with pronase E or trypsin. Initial work on hemoglobin with modified Edman-degradation revealed amine alkylation of terminal valine. Several works is based on or enzymatically cleaved with identification of adducts. The enzymatic cleavages is done by various enzymes from pronas E (complete digestion into individual amino acids), pepsin (cleavage between hydrophobic and aromatic amino acids such as phenylalanine, tryptophan, and tyrosin) and with specific cleavage trypsin (carboxyl side of the amino acids lysine or arginine).

Edman degradtion of terminal valine in blood hemoglobin: Fidder studied hemoglobin in human blood after in vitro exposure with mustard gas and in vivo to guinean pigs. The N-terminal valine was released with Edman degradation followed by derivatisation with HFBA and GC-MS analysis further developed to the use of modified Edman reagent pentafluorophenyl isothiocyanate. Using negative chemical ionization the mustard adduct to the nitrogen could be identified and detected at 30-100nM levels in blood [74, 77, 78]. This site may be used as a biomarker for mustard exposure in rat hemoglobin as the N-terminal valine adduct which were observed up to 28 days after exposure [74]. Black demonstrated both β-chain and α-chain N-terminal valine HETE-adducts as the key site for biological marker of sulfur mustard poisoning[76].

Analysis of mustard alkylation after enzymatic digestion of hemoglobin and serum albumine with Pronas E: The enzymatic cleavages are done by various enzymes. Using pronas E will give almost complete digestion into individual amino acids. This hydrolysate can be analyzed with GC-MS after derivatisation with MTBSTFA or analysed directly with LC-APCI-MS alternatively after derivatisation with pentafluoreobenzylisocyanate [76, 79]. The N-terminal Valine is identified as a key alkylation site in both hemoglobin chains will be identified as HETE-alkylated dipeptide. Furthermore, confirmed alkylated sites identified in Pronas E digest were histidine, aspartic acid, cysteine, glutathione and tentatively identified lysine, glutamic acid and tryptophan [76]. A fluorenylmethyl chloroformate (Fmoc-Cl) derivatisation procedure for sensitive detection of alkylated histidine with LC-MS /MS detection were set up by Noort [75]. A procedure for pronas E digestion of human serum albumin followed by LC-MS/MS identification were done for sulfur mustard alkylated cysteine were a tripeptide of (S-HETE)Cys-Pro-Phe and tested for Iranian victims from the Iran-Irak war. The modified site was cysteine C34 [74, 80].

Analysis of mustard alkylation after enzymatic digestion of hemoglobin and serum albumin with pepsin: Pepsin performs enzymatic cleavage between hydrophobic and aromatic amino acids like phenylalanine, tryptophan, and tyrosin. Immobilized pepsin has been used in an on-line system for digestion of isolated human serum albumin aiming for rapid detection of potential HETE-cysteine conjugate (C34) reported above by Noort [80]. The protein is isolated by affinity chromatography over HiTrap Blue cartridge of plasma from blood after centrifugation. After 5min on-line digestion was the HETE-modified site (Cysteine C34) identified in a 12 amino acid long peptide. However, the most pronounced were a glutamic acid conjugate HETE-A(E)VSK peptide were recommended as diagnostic fragment sulfur mustard exposure suitable for detection of low µM detection [81].

Analysis of mustard alkylation after enzymatic digestion of hemoglobin and serum albumin with Trypsin: Digesting with trypsin (carboxyl side of the amino acids lysine or arginine) leaves larger fragment that have suitable properties for analysis with LC-ESI-MS/MS. Analysis of conjugation in hemoglobin identified conjugation towards terminal valine and to histidine and glutamic acid. Diagnostic ion m/z 105 $[HOCH_2CH_2SCH_2CH_2]^+$ are used to identify alkylated peaks. Using radioactive and heavy label easify the identification. Ester to glutathione were identified after tryptic digestion of hemoglobin (and corresponding finding for PronasE disgetion) but not before[76]. In tryptic digestion of hemoglobin alkylated esters may go

towards aspartic acid, and glutamic acid be hydrolyzed [76, 82]. Alkylated cysteine HETE-adduct was identified in human serum albumin by Noort et al [78, 80].

Skin analysis: Noort s suggested mild alkaline hydrolysis of keratine protein in order to release sulfur mustard HETE-adducts towards glutamic and aspartic acid followed by derivatisation with fluorine containing reagent and identification with GC-NCI MS [78].

Bacterial degradation of sulfur mustard

Mustard gas bombs dumped at sea or buried in soil will be toxic for the present microorganisms were micromycetes and actinomycetes are more sensitive as compared to bacteria's [83]. Bacterium collected at the mustard dumpsites in the Baltic Sea showed reduced diversity of microbiota and in laboratory they were able to dechlorinate sulfurmustard and use it as the source of carbon. The metabolic pathway is hydrolysis to thiodiglycol followed by oxidation of the hydroxyl groups to [(2-hydroxyethyl)thio]acetic acid and further to thioglycolic acid **Figure 14**. The final metabolic products are then described as sulfate and acetic acids [3].

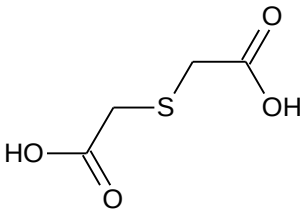


Figure 14. Thiodiglycolic acid, a sulfur mustard bacterial degradation product.

4. Nerve agents

Research carried out in the 1930s to develop new insecticides led to the discovery of a number of highly toxic organophosphours compounds. Some of them were weaponized and classified as nerve agents due to its action in the nervous system. The initial progress occured in Germany and the compounds are denoted G-series *e.g*. tabun, sarin, soman and cyclosarin.

This was followed by research in England were an insecticide Amiton reached the market in the fifties before it was withdrawn and studies of organophosphate esters substituted with 2-aminoethanols further developed to V-series of nerve agents *e.g.* VX and VR. VX is rather stable in water but will eventually hydrolyse, one of these products are also toxic EA2192. This compound will likely persist for longer time in salt water and has to be included when environmental effects will be taken into account [51, 66, 84, 85]. The V-types of compounds are not expected to be dumped in the Baltic Sea.

Today they are under the regime of the CWC (1997) with a total ban and obligation of destructions of existing chemical weapons and product facilities. This has resulted in a less likelihood that these compounds should be used in a conflicted. However, there are concerns that these compounds could become in a part of a terrorist attack and there are still needs for methods to handle such an incident like the Aum Shinrikyo incident in Tokyo 1995 were twelve people were killed and several thousand had to seek medical treatment.

Analytical methods for rapid screening and structure determination of the agents and their degradation products are needed in the management of an incident in order to characterize contaminated area, exposure and to provide forensic evidence. Nerve agents have a short life span in the environment and they are rapidly hydrolyzed to stable phosphonic acids, which are used as markers.

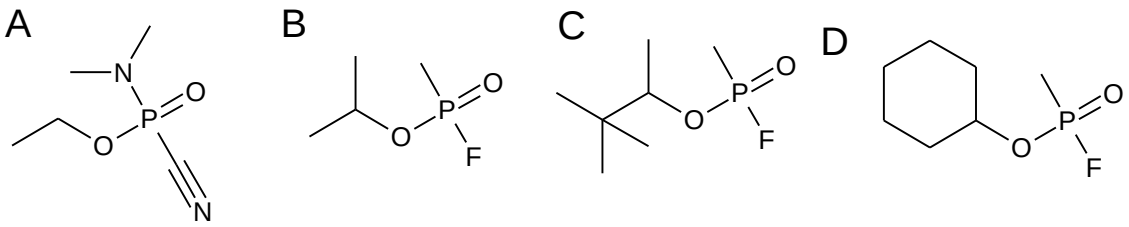


Figure 15 G-agents, A) tabun, B) sarin, C) soman D) cyclosarin

Alkylphosphonic acids with methyl-, ethyl- or propyl-group attached to the phosphourous atom are considered as suitable markers for nerve agent exposure in environmental and biomedical samples. However, the phosphoramidate tabun with no alkylchain attached to the phosphourous atom will degrade to ethyl phosphoric acid which can originate from several compounds with legal use and further to phosphoric acid **Figure 16.**

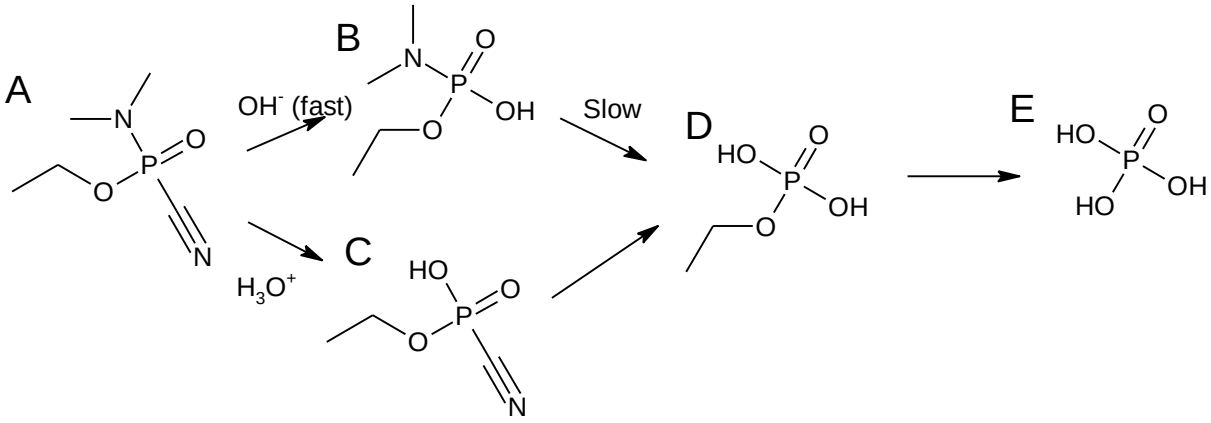


Figure 16 Hydrolysis process for tabun.

4.1.Preparation of sample prior GC-MS analysis of phosphonic acids

Liquid-liquid Extraction: According to the standard protocol [2], the aqueous sample is acidified in order to protonate the alkylphosphonic acids (pKa aprox. 2.5) and are extracted twice with organic solvent (dichloromethane). The combined extracts are dried prior derivatisation and analysed with GC-MS. This procedure works for at least for the more non-polar phosphonic acids. Most efficient for phosphonic acids is to partitioning against organic solvent for removal of matrix followed by concentration of water phase. If silylation is done prior analysis for GCMS divalent cations has to be removed with cation exchange.

Soils were extracted in basic aqueous conditions (0.1M NaOH) and trapped on SAX column. With analysis with CE-ion trap MS the yields of MPA, EMPA, IMPA and PinMPA were 67% or better. (Spiking levels approx 150µg AMPA/2 gr soil) [86].

Samples (serum) were acidified with sulphuric acid and partitioned against isobutanol/toluene which for IMPA yields of 70-100% [87].

Solid Phase Extraction: There are two strategies in order to isolate phosphonic acids, with hydrophobic interaction or ion exchange. In the most recent work is balanced hydrofobic/hydrophilic Oasis HLB preferred. [88]. However, if combined with isolation of TDG compounds will ENV+ be a better choice (own results, Read at 3CWA workshop Helsinki)

Ion exchange will probably work poorly in brackish /salt water but is useful for less salty samples. There are three strategies for ion exchange isolation of the phosphonic acids. 1) Elution and solvent exchange followed with derivatisation [86] 2) Elution of acid and ion pair reagent that will work as on column methylation reagent [89, 90] 3) solid phase derivatisation releasing the acid with silylation [91].

Solid Phase Mikro-Extraction: Headspace SPME extraction of nerve agents followed by desorption electrospray ionization mass spectrometry (DESI-MS) was set up for forensic analysis in terrorist scenario. Samples were introduced to a head space vial and heated 40-60°C during head space sampling. The SPME fiber is then exposed to electrospray plume and ionization will occur. The sample was contaminated with µg amount studying sarin, tabun, mustard gas, cyclosarin, VX and soman and headspace SPME sampling followed with MS was successfully performed[92].

Derivatisation

The nerve gas hydrolysis products, the phosphonic acids, require derivatisation prior separation with GC. Most common procedures are silylation, forming tertbythyldimethylsilyl- or trimethylsilyl-derivatives, methylation and esterification of pentafluorophenylgroup. The silylation or methylation is normally done with environmental samples prior analysis with GC-EI-MS and derivatisation with fluorinated reagents prior analysis requiring high sensitivity, *e.g*. biomedical samples, with GC-NegCI-MS.

Silylation: Analysis of silylated phosphonic acids in GC-quadrupole MS systems will give a full scan LOD 300-500pg injected and in SIM LOD 30-60pg [23].

Even moderate amount of divalent cations will hindered the silylation of phosphonic acids. In order to overcome this problem were SPE with a cation exchange column performed. This is routinely done prior silylation with MTBSTFA and BSTFA, (FBB) [8]. This may be done with H^+ as counter ion with the exception for phosphor amidates which will hydrolyze in acid conditions. In that chase will exchange from H^+ to Na^+ be required. The Na^+ will not interfere with silylation.

Methylation: Methylation has historically been done with diazomethane but due it's to carcinogenic and explosive properties attempt has been made to replace it with trimethylsilyldiazomethane [93] and thermally assisted on column methylation with quaternary reagents, trimethylphenylammonium hydroxide and trimethylsulfonium hydroxide [89, 90, 94, 95].

Optimized method for quantification with GC-MS/MS after methylation with etheral diazomethane has been done for urine after solvent exchange without taking the sample to dryness with azeotropic sovent exchange with acetonitrile. The method LOD was 4µg/L for methylalkyphosphonic acids from sarin, soman, cyclohexylsarin and VX and for O-ethylphosphoric acid 20µg/L.

Using in injector methylation of isopropylmethylphosphonic acid with quaternary agent trimethylphenylamin were the LOD in 2µg/L water in an sector mass spectrometer while Sega analysed with flame photometric detection of samples from ground water LOD 3-9 µg/L (IMPA and EMPTA analyzed) [90, 94].

Flourinated derivatives: In order to detect trace amounts the derivatisation with fluorinated derivatives is preferred in combination with GC-Neg-CI MS in order to obtain high sensitivity. However, the procedures has often low yield when analyzing low amount samples. The choice of method has been with the reagent pentafluorobenzylbromide or pentafluorobenzylchloride. However, it is recommended to use SPE florisil purification in order to avoid deterioration of GC properties by undesired by products from the derivatisation reaction [88, 96].

The use of pentafluorophenyl diazomethane, 3,5-di(trifluoromethyl)phenyldiazomethane and 2,4-di(trifluoromethyl)phenyldiazomethane has been described. Work is in progress in order to investigate explosive characteristic for safe handling and usefulness in low concentration samples [97].

Analysis with GC

Analysis with GC combined with MS will give identification required and are routinely done. Quantification will be possible to obtain with several detectors analyzing the phosphorus heteroatom. In mass spectrometry derivatisation incorporating fluorine is desired for analysis with GC–MS with negative chemical ionization.

Analysis with LC-MS

The interface of LC and MS is today dominated with electrospray (ESI) alternatively atmospheric chemical ionization (APCI). The usage is to large extent overlapping with advantage to ESA with larger and "naturally" ionisable groups while the advantage of APCI is with more stable and neutral molecules. The LC-MS analysis is in the field of CWA analysis seen as complement to GC-MS in the screening of regulated substances or alternatively used for directed analysis without derivatisation [66]. Earlier system with frit-FAB, particle beam and thermospray are today mostly considered obsolete.

In order to achieve screening of nonderivatised sample LC-MS was set up as a complementary technique for identification of phosphonic acids with GC-MS. LOD concentrations in aqueous solution were less than 50ng/ml (250pg injected 1998). Positive ESI yielded better sensitivity compared to APCI (factor 2). For ESI the positive spectra $[M+H]^+$ were more sensitive as compared to negative spectrum $[M-H]^-$ and they recommended formic acid as modifier. Spectrum contains suitable screening ions and in MS/MS transition from the protonated pseodomolecular ion and suitable daughter ions. Reversed phase hydrophobic interaction with *e.g.* combined C8, C18 or C18 is recommended. [23, 84]. VX and its degradation products and stabilizer were analyzed with capillary column LC-ESI-MS for identification purposes. Identification in ESI more complex adducts formation and more sensitive for ion suppression effects from the matrix as compared to APCI. Expected LOD for tandem MS/MS 10mg/kg 0.1mg/kg for quantification purposes.

Analysis with LC-ESI-MS/MS (tripplequadrupole) yielded an absolute LOD of 10pg (negative) and 2pg (positive) of IMPA. The analysis of serum samples from Japanese victims from sarin attacks in Japan revealed levels 2-135ng/mL (µg/L.) using positive ESI-mode. (Isolation by partitioning acidified water/isobutanol-toluene). Negative ESI analysis of serum samples revealed only one single peak originated from the phosphonic acid with a LOD of the method at 4ng/mL, thus demonstrating high selectivity of the acids. The overall recovery was 70-110% [87].

For forensic application was rapid water extraction of environmental and indoor samples performed followed by LC-ESI MS analysis for the simultaneously identification of active agents such as sarin and VX and their hydrolysis products µg/g contaminations [98]. Also the extraction from water with hollowfiber was demonstrated prior LC-ESI-MS analysis.[99].

Derivatisation in LC-MS for phosphoic acids has been tested with the quaternary amine trimethylphenylamonium added post column to LC-APCI-MS system. The derivatisation of EA2192 and methylphosphonic acids was complete when analyzing 145µg/mL concentrations without make any drastic improvement of the analysis[49].

4.2. Biological samples

Nerve agents irreversible inhibit the enzyme acetylcholinesterase in the synapse and thereby block the turnover of acetylcholine. This prevents the muscles from relaxing and causes muscle contraction. Measurable adduct formation in biomedical samples of nerve agents will be traceable to serine esterases *e.g.* buthylcholine esterase (ref) and other identified binding to macromolecules such as albumin. However most of the nerve agents will *in vivo* hydrolyze as in aqueous samples but *in vivo* also enzymatically catalyzed by phosphoric triesterhydrolases and after exposure there will be possibility to detect the corresponding alkylphosponic acid in blood and urine (Black2010).

The adduct formation towards cholinesterase is towards an serine in the active site can after nerve gas exposure be traced in measureable amount from acetylcholinesterases in red blood cells or from buterylcholinesterases in plasma. Since the buterylcholinesterases occurs in higher concentrations it will

most often be target for analysis (AChE 3nM BuChE 80nM) [100] with serine-198 as the target for adduct formation[101]. The O-alkylchain on the phosphonic acid is hydrolyzed in a process called ageing with agent depending rate and especially soman is aged rapid. The ageing process for tabun occurs with O-dealkylation and deamidation. Deamidation is also a problem if acid conditions are used under the work up procedure [102-104].

The phosphonyl-adducts identified in serum albumin at tyrosin (residues 411 in human) are more stable against the ageing process and also towards oxime reactivation. However, the formation of this adduct are more pronounced with the more reactive G-agents as compared to the V-series and it is less sensitive biomarker at low dose exposure [103].

In order to obtain adduct formation corresponding to nerve gas action analogues were created with thiocholine replacing fluoride and cyanide leaving group[105]. This provides a tool for producing nerve agent adducts in proteins for investigation whiteout handling the actual high toxic substances in *in vitro* systems.

Hydrolysis products

The analysis of phosphonic acids in complex matrixes may preferably be done with isotope dilution technique with heavy labeled (deuterium published, (^{13}C, ^{15}N, ^{33}P potential)) internal standards in **figure 17.** Driskell analyzed urine by exchanging solvent to acetonitrile with azeotropic distillation followed by centrifugation to remove insoluble constituents. Taking the sample to dryness will result in yield losses. Derivatisation was done with diazomethane. The analysis was done with GC-MS/MS resulting in an extraction yield 31-47%, and quantification range of alkylphosphonic acids 2-200µg/L (GA 17-1000µg/L)[85].

Riches concentrated alkylphosphonic acids on Oasis HLB (yield EMPA 24%, iPrMPA, EEPA, iBMPA and CMPA 85-90%) with an overall yield after derivatisation(EMPA 17 %, others 45-60%). The samples were taken to dryness and redissolved prior derivatisation with pentafluorophenyl bromide. GC-neg CI-MS were performed in SIM mode on a quadrupole instrument. LOD of the instrument were 0.06ng/mL (µg/L) and assigned analytical range was 0.5-10ng/mL (µg/L)[88].

Instrumental performances that are expected are GC-MS has a LOD in low ppb range while GC-MS/MS and will LC-MS/MS perform in sub ppb LOD.

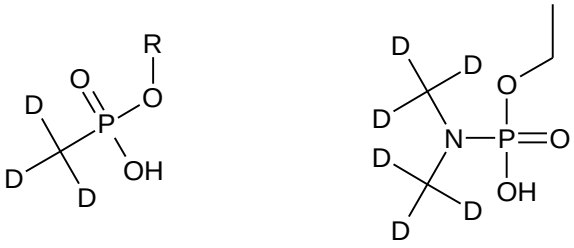


Figure 17; Strategies for deuterium labeling of phosphonic acids for the use as internal standards

Samples (serum) were acidified with sulphuric acid and partitioned against isobutanol/toluene which for IMPA yields of 70-100%. Analysis with LC-ESI-MS/MS (tripplequadrupole) yielded an absolute LOD of 10pg (negative ESI) and 2pg (positive ESI) of IMPA. The analysis of serum samples from Japanese victims from sarin attacks in Japan revealed levels at 2-135ng/mL (µg/L) using positive ESI-mode. Negative ESI analysis of serum samples revealed only one single peak originated from the phosphonic acid with a LOD of the method at 4ng/mL, thus demonstrating high selectivity of the acids. The overall recovery was 70-110% [87].

Combined Trypsin digestion and Phosphatase treatment

After the sarin attack in Tokyo subway a method for analyzing sarin in acetylcholinesterase as conjugated phosphonic acids in erythrocytes was developed. The erythrocytes were isolated and lysed followed by enzymatic treatment. Initially are the proteins digested with trypsin followed by alkaline phosphatase in order to hydrolysis of the phosphonic part. The overall procedures including incubations were 3-4 days long. The phosphonic acid is isolated and analyzed on GC-MS [102, 106].

Flouridat reactivation

Nerve agent adducts against cholinesteases can be released with fluoride ions generating the corresponding fluoridate. Plasma is diluted with 200mM sodiumacetate buffer pH 3.5 and fluoride activated reactivation is performed with 250mM KF at 15min, 25°C. Effective reactivation is performed in a final pH 4-6. Lower pH will increase ageing (hydrolysis of O-alkylchain from the phosphono-moiety forming *e.g.* methylphosphonic acid). The aged phosphonic acid will not be reactivated with this method. Formed fluoridates were extracted with SPE (C18 or Nexus) and eluted in ethyl acetate. The ethyl acetate phase is then applied on a thermal desorption tube. Thermal desorption were performed on two dimensional GC and NPD detector or GC.MS with chemical ionization with methane in positive mode. The *in vitro* half-life is reported to 14h (GB)-63h (GA) while the *in vivo* phosphofluoridate regeneration of GF and GB varied between 14-56 days [100, 107]. Replacing thermal desorption with large injections on PTV injector on GC-MS was done by Dabisch et al [108].

In order to convert VX to it fluoridate analog SPE C18 isolated VX were injected over a silverflourid pad in a thermal desorption system. This converted VX to its fluoridate analog [109]. Silverfluoride conversion tubes are used in field instrument to convert VX to its corresponding fluoridate since VX is too high boiling for the system (www.inficon.com).

Enzymatic digestion of buturylcholinesterase for the identification of phosphonate adducts

The adduct formation of nerve agents by phosphonylation of is towards a serine in the enzyme active site (serine-198 in BuChE). This can be analyzed with LC-MS/MS as a modified peptide after enzymatic digestion. The conjugated phosphonyl-group undergo aging, a dealkylation were the diagnostic O-alkylchain is lost. This process can be studied with LC-MS/MS and ageing rate varies whit agent but can be rapid for especially soman [110]. Human butyrylcholinesterase is isolated from plasma with procainamide gel affinity and the eluted protein is concentrated on a centrifuge 100kD cut off filter. Initially enzymatic digestion were tested with trypsin, thermolysine, protonase and pepsin yielding 27 or 29, 6, 7 and 12 or 9 amino acid long peptides, respectively. The pepsin protocol was recommended for enzymatic digestion with the conjugated organophosphorous adduct ending up in a nonapeptide. Identification was done on a Q-TOF instrument [101]. The method was further automated with on-line pepsin-digestion in a stop-flow manner in LC-MS/MS analysis. This set up was also tested for mustard adducts [81]. For trace analysis was a LC-MS/MS method based on predicted SRM transitions developed. In a generic method organophosphorous adduct is replaced with the 2-(3-aminopropylamino) ethanol and analyzed with LC-MS/MS **Figure 18** [111].

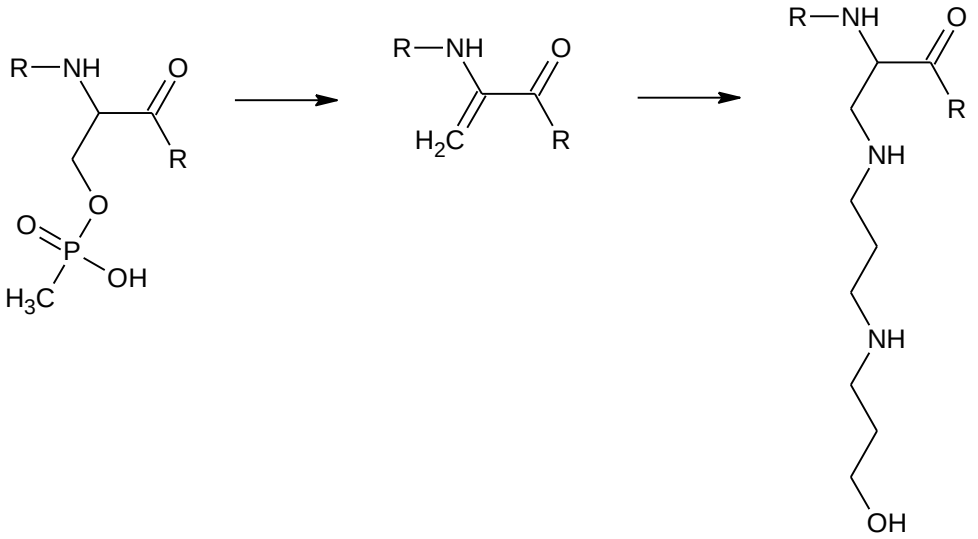


Figure 18; Treatments of pepsin digest of BuChE will result in a phosphanated nonapeptide. This can be analyzed directly with LC-MS/MS or transferred to a generic marker. Barium hydroxide will release the phosphonate forming a dehydroalanine that can be reacted with2-(3-aminopropylamino) ethanol.

Enzymatic digestion of albumin for the identification of phosphonate adducts

Incubating human blood plasma with the nerve agent's sarin and soman, labeled with deuterium or carbon-^{13}C, lead to identification of phosphonylated-tyrosin. The identification were done on a pronase E digest and suggested to be towards human serum albumin. This fraction was larger than the conjugation towards buturyl choline esterase[112]. Extended studies that also included cyclosarin, tabun and VX demonstrated this phosponylation (phosphorylation from tabun) rather resistant towards ageing processes and oxime reactivation. The analysis was done with LC-ESI-MS/MS after SPE C18 alternatively C18 purification after pronase E digestion to individual amino acids. Replacing Pronase E with protease type XIV doubled the yield. Digestion with trypsin resulted in a tripeptide and the identification of albumin as the origin were considered tentatively [103, 113]. Organophosphorus adduct in human serumalbumin were demonstrated to occur at tyrosine-411 with MALDI-TOF and QTRAP-MS on peptides after pepsin digestion [114].

4.3.Related agents

There are some chemical weapons agent that rather rapidly will degrade in contact with water and are therefore problematic to detect with chemical analysis.

Phosgene; Phosgene was loaded into weapons by USA, It is an important intermediate in plastic industries making polyurethane. Phosgene hydrolyses in seawater to chloride ions and carbon dioxide. [51]. This compound will probably not be to any problem in a marine environment.

Cyanogen Chloride; Cyanogen chloride will in contact with water within days hydrolyse to cyanic acid and further to ammonia and carbon dioxide. [51]. Released cyanide-chloride and its hydrolysis product cyanic acid will be toxic to the environment.

Hydrogen Cyanide; Volatilization and biodegradation are considered to be the most important environmental processes for the loss of cyanide in shallow water. But there are no studies focusing on deeper water. Released cyanide will be toxic to the environment,[51]

5. Target chemicals for the CHEMSEA project.

This review of analytical methods was done within the CHEMSEA project prior the analysis of samples taken in the Gotland deep in 2012. Analysis will be done on environmental samples taken close to objects suspected to contain CWA related material. The choice of analytical methods and suitable marker chemicals to be used in analysis is based on this review and VERIFIN experience on analysis of samples taken during the MERCW-project in the Bornholm deep. The chemicals that were discussed within the CHEMSEA project and its chemical / physical data in **Table 1** and the chemicals decided to be used as screening target in environmental analysis in **Table 2.** The basic principle for sample preparation is presented in **Figure 19**. Methods for environmental analysis are tested in an interlaboratory calibration study between VERIFIN Finland, FOI Sweden and MUT Poland.

The result from the environmental analysis will be ready and presented in spring 2013. Target chemicals for the analysis of biological samples are yet under discussion.

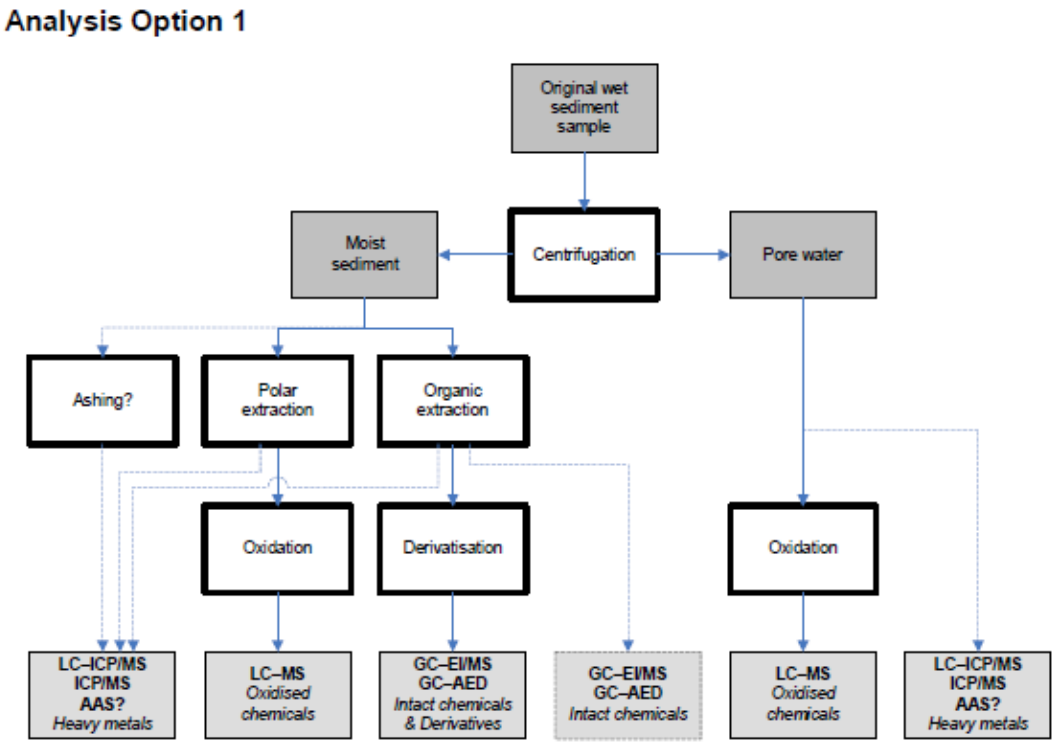
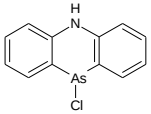
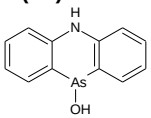
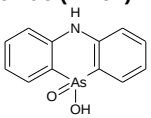
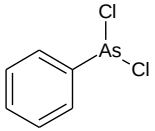
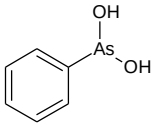
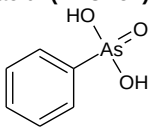


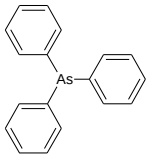
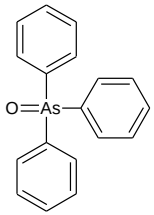
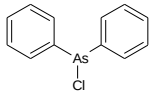
Figure 19 CHEMSEA basic principle schedules for analysis of the target chemicals

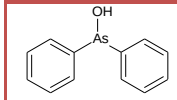
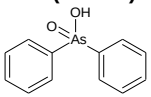
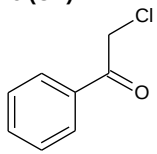
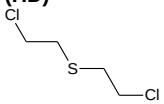
Table 1. Estimated and experimental physico-chemical properties of the studied chemicals. **CAS nr = Chemical Abstract Service number, MW = Molecular weight,MP = Melting point, BP = Boiling Point, Log Kow = log Octanol-Water partitioning coefficient, log D = log Octanol-Water** distribution coefficient (at pH 8), Log Koc = log Organic Carbon-Water partitioning coefficient, BCF = bioconcentration factor

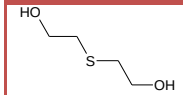
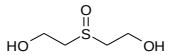
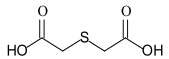
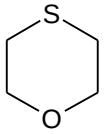
[a] estimated values derived from EPISuite 4.10 software (U.S. Environmental Protection Agency, Washington, DC, USA)
[b] experimental values were available in the EPI Suite experimental database
[c] estimated values from Advanced Chemistry Development (ACD/Labs) Software V11.02 (available from SciFinder)
[d] experimental values available from SciFinder (de flesta från Syracuse Research Corporation of Syracuse, New York)

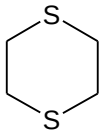
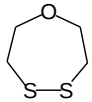
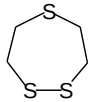
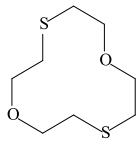
	CAS nr	MW (g/mol)	MP (°C)	BP (°C)	Density (g/cm^3)	pKa	Solubility in water (mg/L)	Log Kow	Log D (pH 8)	Log Koc	BCF (PH 8)
Adamsite (DM) 	578-94-9	277.59	124[a]/ 195[b]	368[a]/ 410[b]	-	-3.3[c]	0.45[a]	4.1[a]		3.4[a]	-
Phenoarsazin-10(5H)-ol 	18538-32-4	259.14	147[a]	403[a], 419[c]	-	-1.6, 12[c]	17[a]	2.3[a]		3.4[a]	-
5,10-Dihydropheno-arsazin-10-ol 10-oxide (DMox) 	4733-19-1	275.14	163[a]	403[a] / 365[d] decomp	-	4.0[c]	14[a]	2.3[a]		3.6[a]	-

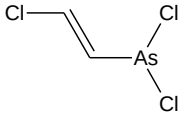
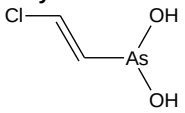
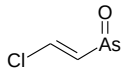
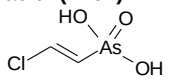
	CAS nr	MW (g/mol)	MP (°C)	BP (°C)	Density (g/cm^3)	pKa	Solubility in water (mg/L)	Log Kow	Log D (pH 8)	Log Koc	BCF (PH 8)
Phenyldichloroarsine (PDCA) 	696-28-6	222.93	0.21[a] / -19[b]	248[a]/ 255[b]	1.65[d]	-	80[a]	3.1[a]		2.8[a]	-
Phenylarsonous acid 	25400-22-0	186.04	40[a]	337[a], 311[c]	-	8.3[c]	48 300[a]	0.03[a]		2.8[a]	-
Phenylarsonic acid (PDCAox) 	98-05-5	202.04	101[a] / 158[d] (decomp)	337[a]	1.76[d]	3.6[c]	38 000[a] , 1000 000[c] (pH 8)	0.03[a] / 0.06[b]	-3.5[c]	3.0[a] , 0[c] (pH 8)	1.0[c]
Triphenylarsine (TPA)	603-32-7	306.24	109[a]/ 61[b]	402[a]/ 360[b]	1.23[d]	-	0.090[a]	6.0[a]		5.5[a]	-

	CAS nr	MW (g/mol)	MP (°C)	BP (°C)	Density (g/cm^3)	pKa	Solubility in water (mg/L)	Log Kow	Log D (pH 8)	Log Koc	BCF (PH 8)
											
Triphenylarsine oxide 	1153-05-5	322.24	141[a] / 195[d]	402[a], 468[c]	-	-	0.072[a], 1.5[c]	6.0[a], 3.8[c]	-	5.7[a], 3.42[c]	420[c]
Clark 1 	712-48-1	264.59	45[a]/ 44[b]	334[a]/ 337[b]	-	-	2.7[a]	4.5[a]	-	4.2[a]	-
Diphenylarsinous acid	6217-24-9	246.14	74[a] /171[d]	369[a]/350[c]	-	12.9[c]	330[a]	2.8[a]		4.2[a]	-

	CAS nr	MW (g/mol)	MP (°C)	BP (°C)	Density (g/cm^3)	pKa	Solubility in water (mg/L)	Log Kow	Log D (pH 8)	Log Koc	BCF (PH 8)
											
Diphenylarsinic acid (DPAox) 	4656-80-8	262.14	124[a] / 178[d]	369[a]		4.8[c]	270 / 120 000[c] (pH 8)	2.8[a]	-1.1[c]	4.4[a] /0[c] (pH 8)	1.0[c]
□-Chloroacetophenone (CN) 	532-27-4	154.60	20[a] /57[b]	241[a]/ 247[b]	1.2[d]	-	1640[a], 1500[c]	1.9[a], 1.72[c]		2.0[a], 2.3[c]	12[c]
Sulphur mustard (HD) 	505-60-2	159.07	-18[a]/ 14[b]	210[a]/ 216[b]	1.28[d]	-	606[a], 4100[c]/ 684[b]	2.4[a], 2.1[c]	-	2.4[a], 2.5[c]	25[c]
Thiodiglycol (TDG)	111-48-8	122.18	25[a]/ -10[b]	249[a]/ 282[b]	1.22[d]	-	340 000[a], 371 000[c]/ 1000 000[b]	-0.62[a] / -0.63[b]	-	0[a], 0.99[c]	1.0[c]

	CAS nr	MW (g/mol)	MP (°C)	BP (°C)	Density (g/cm^3)	pKa	Solubility in water (mg/L)	Log Kow	Log D (pH 8)	Log Koc	BCF (PH 8)
											
Thiodiglycol sulfoxide 	3085-45-8	138.18	74[a]	305[a]			1000 000[a]	-2.76[a]		0[a]	
Thiodiglycolic acid 	123-93-3	150.15	110[a]	322[a]			1000000[a]	-1.16[a]		1.13[a]	
1,4-Oxathiane 	15980-15-1	104.17	-28[a] / 30[d]	137[a]/ 147[b]	1.12[d]	-	40 000[a], 23000[c]	0.53[a], 0.77[c]	-	1.3[a], 1.8[c]	2.3[c]
1,4-Dithiane	505-29-3	120.23	6.9[a] / 112[b]	170[a]/ 200[b]	1.14[c]	-	22 000[a], 18000[c] / 3000[b]	1.4[a], 0.11[c] / 0.77[b]	-	2.2[a], 1.4[c]	2.9[c]

	CAS nr	MW (g/mol)	MP (°C)	BP (°C)	Density (g/cm^3)	pKa	Solubility in water (mg/L)	Log Kow	Log D (pH 8)	Log Koc	BCF (PH 8)
											
1,4,5-Oxadithiepane 	3886-40-6	136.23	27[a]	198[a]			4700[a]	1.49[a]		1.55[a]	
1,2,5-Trithiepane 	6576-93-8	152.29	40[a]	227[a]			750[a]	2.34[a]		2.42[a]	
1,7-Dioxa-4,10-dithiacyclododecane 	294-95-1	208.34	75[a]	300[a]			7700[a]	0.84[a]		2.0[a]	

	CAS nr	MW (g/mol)	MP (°C)	BP (°C)	Density (g/cm^3)	pKa	Solubility in water (mg/L)	Log Kow	Log D (pH 8)	Log Koc	BCF (PH 8)
Lewisite I 	541-25-3	207.32	-13[a]/-1.2[d]	156[a]/197[d]	1.88[c]	-	262[a]	2.6[a]		2.0[a]	
Vinylarsinous acid 	85090-33-1	170.43	52[a]	270[a], 256[c]		7.68[c]	155000[a]	-0.47[a]		2.0[a]	
2-Chlorovinylarsinic oxide 	3088-37-7	152.41	18[a]	121[a]			1640[a]	1.94[a]		1.86[a]	
2-Chlorovinylarsonic acid (L1ox) 	64038-44-4	186.43	84a	290a			130000a	-0.47a		2.2a	

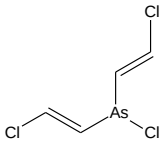
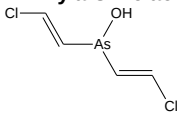
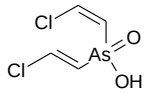
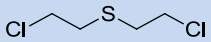
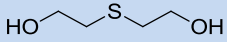
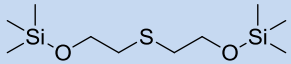
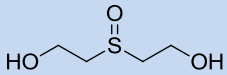
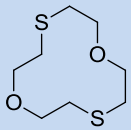
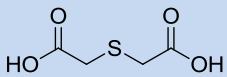
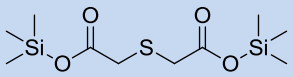
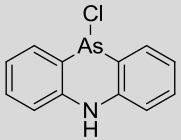
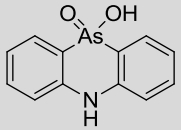
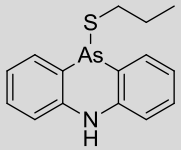
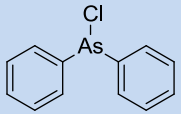
	CAS nr	MW (g/mol)	MP (°C)	BP (°C)	Density (g/cm^3)	pKa	Solubility in water (mg/L)	Log Kow	Log D (pH 8)	Log Koc	BCF (PH 8)
Lewisite II 	40334-69-8	233.36	10[a] / 0.1[d]	204[a]/230[d]	1.87[d]	-	30[a]	3.5[a]		2.6[a]	
Divinylarsinic acid 	157184-20-8	214.91	42[a]	259[a], 229[c]			3510[a]	1.79[a]		2.6[a]	
Bis(2-chlorovinyl)-arsinic acid (L2ox) 	157184-21-9										

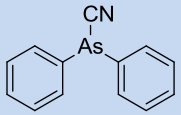
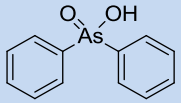
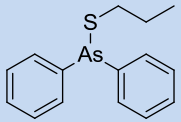
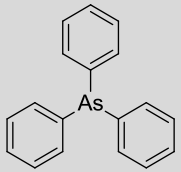
Table 2; Target chemicals for screening in CHEMSEA project

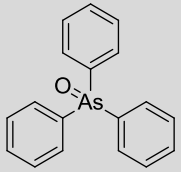
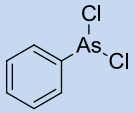
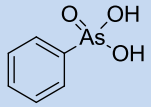
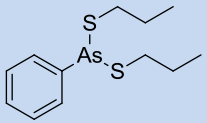
PrSH = propan-1-thiol; **BSTFA** = (N,O-bis(trimethylsilyl)trifluoroacetamide

#	Chemical (acronym) CAS	Structure	Description	GC-based			LC-based	
				intact	PrSH	BSTFA	intact	H_2O_2
1	Sulphur mustard (H) 505-60-2		Dumped CW agent	x				
1.1	Thiodiglycol (TDG) 111-48-8		Hydrolysis product of **1**				x	
1.1S	Bis(2-siloxyethyl)sulfide 20486-03-7		BSTFA derivative of **1.1**			x		
1.1O	Thiodiglycol sulfoxide 3085-45-8		Oxidation product of **1** (either natural or with H_2O_2)					x
1.2	1,4-Dithiane 505-29-3		Degradation product of **1**	x				

#	Chemical (acronym) CAS	Structure	Description	GC-based			LC-based	
				intact	PrSH	BSTFA	intact	H_2O_2
1.3	1,4-Oxathiane 15980-15-1		Degradation product of **1**	**x**				
1.4	1,4,5-Oxadithiepane 3886-40-6		Degradation product or by-product of **1**	**x**				
1.5	1,2,5-Trithiepane 6576-93-8		Degradation product or by-product of **1**	**x**				
1.6	1,7-Dioxa-4,10-dithiacyclododecane 294-95-1		Degradation product or by-product of **1**	**x**				
1.7	Thiodiglycolic acid (TDGA) 123-93-3		Bacterial metabolite of **1.1**				**x**	
1.7S	Bis(trimethylsilyl) 2,2'-thiodiacetate 20486-03-7		BSTFA derivative of **1.7**			**x**		

#	Chemical (acronym) CAS	Structure	Description	GC-based			LC-based	
				intact	PrSH	BSTFA	intact	H_2O_2
2	*Adamsite (DM)* *578-94-9*		*Dumped CW agent*	***Not analysed as such***				
2O	5,10-Dihydrophenoarsazin-10-ol 10-oxide 4733-19-1		Oxidation product of **2** and all of its degradation products (either natural or with H_2O_2)					**x**
2S	10-(propylthio)-5,10-dihydro-phenarsazinine 7269-24-1		Derivative of **2** and all of its degradation products		**x**			
3a	*Clark I (DA)* *712-48-1*		*Dumped CW agent. Also component in dumped arsine oil.*	***Not analysed as such***				

#	Chemical (acronym) CAS	Structure	Description	GC-based			LC-based	
				intact	PrSH	BSTFA	intact	H_2O_2
3b	*Clark II (DC)* *23525-22-6*		*(Possibly) dumped CW agent*	***Not analysed as such***				
3O	Diphenylarsinic acid 4656-80-8		Oxidation product of **3a** and **3b** and all of their degradation products (either natural or with H_2O_2)					**x**
3T	Diphenylpropylthioarsine 17544-92-2		Derivative of **3a** and **3b** and all of their degradation products		**x**			
4	Triphenylarsine (TPA) 603-32-7		Component in dumped arsine oil	**x**				

#	Chemical (acronym) CAS	Structure	Description	GC-based			LC-based	
				intact	PrSH	BSTFA	intact	H_2O_2
4O	Triphenylarsine oxide 1153-05-5		Oxidation product of **4** and all of its degradation products (either natural or with H_2O_2)					**x**
5	*Phenyldichloroarsine (PDCA)* *696-28-6*		*Dumped CW agent. Also component in dumped arsine oil.*	***Not analysed as such***				
5O	Phenylarsonic acid 98-05-5		Oxidation product of **5** and all of its degradation products (either natural or with H_2O_2)					**x**
5T	Dipropyl phenylarsonodithioite 1776-69-8		Derivative of **5** and all of its degradation products			**x**		

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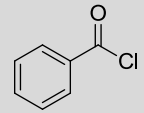
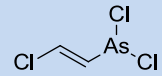
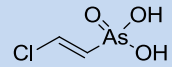
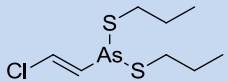
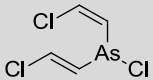
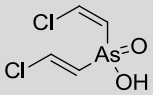
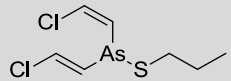
#	Chemical (acronym) CAS	Structure	Description	GC-based			LC-based	
				intact	PrSH	BSTFA	intact	H_2O_2
6	α-Chloroacetophenone (CN) 532-27-4		*Dumped CW agent*	**x**				
7	*Lewisite I (L1)* *541-25-3*		*Dumped CW agent*	***Not analysed as such***				
7O	2-Chlorovinylarsonic acid 64038-44-4		Oxidation product of **7** and all of its degradation products (either natural or with H_2O_2)					**x**
7T	Dipropyl (2-chlorovinyl)arsonodithioite 677354-97-1		Derivative of 7 and all of its degradation products		**x**			
8	*Lewisite II (L2)* *40334-69-8*		*Dumped CW agent*	***Not analysed as such***				
8O	Bis(2-chlorovinyl)arsinic acid 157184-21-9		Oxidation product of **8** and all of its degradation products (either natural or with H_2O_2)					**x**

#	Chemical (acronym) CAS	Structure	Description	GC-based			LC-based	
				intact	PrSH	BSTFA	intact	H_2O_2
8T	Bis(2-chlorovinyl) propylthioarsine 677355-04-3		Derivative of **8** and all of its degradation products		**x**			

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6. Internet Resources

www.opcw.org; Official Homepage for the organization for the prohibition of chemical weapons.

www.imo.org;. IMO – the International Maritime Organization – is the United Nations specialized agency with responsibility for the safety and security of shipping and the prevention of marine pollution by ships. Convention on the Prevention of Marine Pollution by Dumping of Wastes and Other Matter (http://www.imo.org/About/Conventions/ListOfConventions/Pages/Convention-on-the-Prevention-of-Marine-Pollution-by-Dumping-of-Wastes-and-Other-Matter.aspx)

www.helsinki.fi/verifin; The hompage of the Finnish Institute for Verification of the Chemical Weapons Convention. Via this link the "Recommended Operating Procedures for Analysis in the Verification of Chemical Disarment" could be ordered.

www.chemspider.com; ChemSpider is a free chemical structure database providing fast access to over 25 million structures, properties and associated information. It is owned by the Royal Society of Chemistry in Great Britain.

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Vol. Federal office for scentific, technical and cultural affairs. Federal Ministry of Social Affairs, Public Health and Environment, Brussels, Belgium, **2002**.
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