Recommended Operating Procedure (ROP)

Aim of ROP (tick box)

☒ Munition detection or identification	☐ Toxicity
☐ Sampling	☐ In situ exposure studies
☒ Chemical analysis	☐ Bioassays
☐ Bioindicators/biomarkers	

30. Chemical analysis of sea-dumped chemical warfare agents in sediment and pore water

version 1.0

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Scope

This Recommended Operation Procedure (ROP) describes analysis of sea-dumped chemical warfare agents in sediment and pore water samples including sample preparation, analytical methods, and the evaluation of the produced data.

Summary of the method/ROP

This method is based on previously published methods.[1-6] A wet sediment sample is divided into sediment and pore water portions and it is strongly recommended that both portions are analyzed. The ROP utilizes optimized sample preparation for gas chromatography–electron ionization tandem mass spectrometry (GC–EI/MS/MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques with multiple reaction monitoring (MRM), thus allowing for quantification of most target chemicals at concentration level of 1–10 ng/g (= µg/kg, ppb).

The list of target chemicals of this ROP is built upon the different compounds that have been researched during multiple campaigns studying the Baltic Sea sediment samples. The target compound list also contains degradation products of the major chemicals, or their expected degradation and oxidation products.

Safety aspects

General safety aspects conducting laboratory working should be followed when handling any chemicals. Appropriate Safety Data Sheets (SDS's) should be available. Samples should be treated as any other samples containing hazardous chemicals according to laboratory's safety instructions. Proper waste disposal procedures must be followed.

Documentation

When samples are received in the laboratory, each sediment sample must be documented carefully. Documentation must include all the data received from the sampling team, i.e. the date of sampling, the sampling site, the person(s) who did the sampling, and the institute responsible for the sampling. All sample data must be stored electronically. All the samples must be coded electronically in the laboratory's own coding system. Received sample codes and laboratory's codes must be archived, so that they are traceable afterwards. Each sample must have its own specific code and a detailed sample description. The sample codes and the sample descriptions must be identical with the container markings so that every sample can be tracked.

Methods

Target Chemicals

Quantitative chemical analyses of target CWAs are needed to prove presence/absence of dumped chemicals and/or their degradation products in the marine environment and to support for example the risk assessment e.g. for environmental evaluation, marine construction works, fishery, and accumulation in food chain.

Intact target chemicals, which have been dumped in significant amounts in the Baltic Sea, and therefore were analyzed, are:

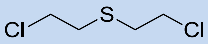
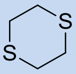
- Sulfur mustard (H),
- Adamsite (DM),
- Clark I (DA),
- Clark II (DC),
- α-Chloroacetophenone (CN),
- Lewisite I (L1) and II (L2),
- Components of arsine oil, which contains TPA and phenyldichloroarsine (PDCA)
- Tabun

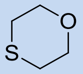
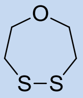
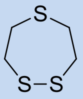
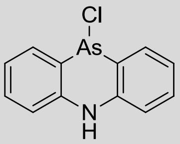
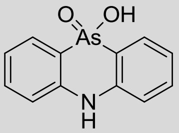
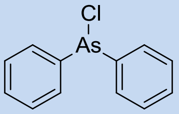
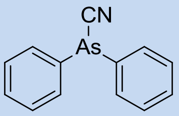
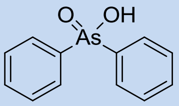
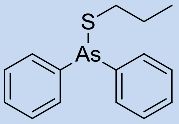
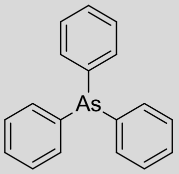
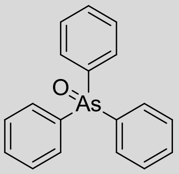
Table 1 summarizes the names of the studied chemicals, their CAS numbers, acronyms, and structures. Some of the degradation products of sulfur mustard (H) (**1**), namely 1,4-dithiane (**1.2**), 1,4-oxathiane (**1.3**), 1,4,5-oxadithiepane (**1.4**), and 1,2,5-trithiepane (**1.5**), were also analyzed, since sulfur mustard (H) (**1**) is readily hydrolyzed and further oxidized in aqueous matrices. In addition, degradation products of Adamsite (DM) (**2**), Clark I (DA) & II (DC) (**3a** & **3b**), Lewisite I (L1) & II (L2) (**7** & **8**), and arsine oil components (**4** & **5**) including their hydrolysis products were analyzed as their respective oxidation products.

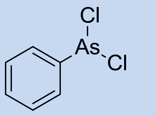
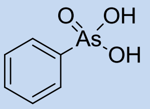
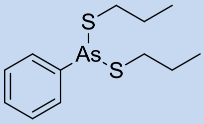
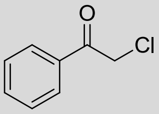
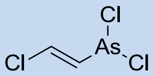
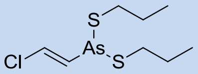
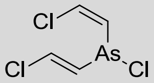
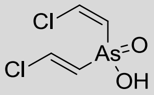
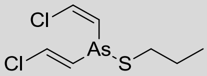
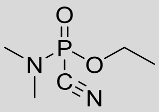
Two types of derivatization methods have been used for the degradation products of the agents:
PrSH – Derivatization using propane-1-thiol: Arsenic-containing chemicals – both chemical warfare agents and degradation products – readily react with thiolsThese derivatives are analysed using GC–MS-based methods.
H_2O_2 – Oxidation using hydrogen peroxide (H2O2): Both arsenic and sulfur-containing chemicals can be oxidised in aqueous samples. The same oxidation reactions can also occur in the sample naturally – probably by exposure to atmospheric oxygen after sampling . These derivatives are analysed using LC–MS-based methods. Often, the oxidation enhances electrospray ionization of the chemical in LC-MS and, therefore, the sensitivity.

The applied main derivatization methods, oxidation with hydrogen peroxide and derivatization with propanethiol, derivatize both the intact warfare agents and their degradation products. Therefore, the results obtained using derivatization are always the sum of all related chemicals.

Table 1. Names, CAS numbers, acronyms, and structures of the studied chemicals.

#	Chemical (acronym) CAS	Structure	Description	GC-based		LC-based	
				Intact	PrSH	Intact	H_2O_2
1	Sulfur mustard (H) 505-60-2		Dumped CW agent	Q			
1.2	1,4-Dithiane 505-29-3		Degradation product of **1**	Q			

1.3	1,4-Oxathiane 15980-15-1		Degradation product of **1**	Q			
1.4	1,4,5-Oxadithiepane 3886-40-6		Degradation product or by-product of **1**	Q			
1.5	1,2,5-Trithiepane 6576-93-8		Degradation product or by-product of **1**	Q			
2	*Adamsite (DM)* *578-94-9*		*Dumped CW agent*			Q*	
2O	5,10-Dihydrophen-arsazin-10-ol 10-oxide 4733-19-1		Oxidation product of **2** and all of its degradation products (either natural or with H_2O_2)				Q
3a¤	*Clark I (DA)* *712-48-1*		*Dumped CW agent. Also component in dumped arsine oil.*	Q*			
3b	*Clark II (DC)* *23525-22-6*		*(Possibly) dumped CW agent*	Q*			
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)				Q
3T	Diphenylpropylthioarsine 17544-92-2		Derivative of **3a** and **3b** and all of their degradation products		Q		
4	Triphenylarsine (TPA) 603-32-7		Component in dumped arsine oil	Q			
4O	Triphenylarsine oxide 1153-05-5		Oxidation product of **4** and all of its degradation products (either natural or with H_2O_2)				Q

5	*Phenyldichloro-arsine (PDCA)* *696-28-6*		*Dumped CW agent. Also component in dumped arsine oil.*	***Will NOT be analyzed, since it is not stable in water matrix***			
5O	Phenylarsonic acid 98-05-5		Oxidation product of **5** and all of its degradation products (either natural or with H_2O_2)				Q
5T	Dipropyl phenylarsonodithioite 1776-69-8		Derivative of **5** and all of its degradation products		Q		
6	α-Chloroacetophenone (CN) 532-27-4		*Dumped CW agent*	Q			
7	*Lewisite I (L1)* *541-25-3*		*Dumped CW agent*	***Cannot technically be analyzed***			
7T	Dipropyl (2-chloro-vinyl)arsonodithioite 677354-97-1		Derivative of 7 and all of its degradation products		Q		
8	*Lewisite II (L2)* *40334-69-8*		*Dumped CW agent*	***Cannot technically be analyzed***			
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)				Q
8T	Bis(2-chloro-vinyl)propylthio-arsine 677355-04-3		Derivative of **8** and all of its degradation products		Q		
9	Tabun 77-81-6		*Dumped CW agent*	Q			

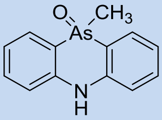
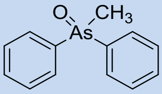
* Quantified intact only if derivative is found

[¤] Clark I will in lower concentrations rapidly degrade in moist or wet conditions. It should, however, be kept on the list of target chemicals as it may be found in sediment samples in some dumpsites where larger amounts have been dumped, e.g. in Skagerrak near ships. Analysis of Clark I may require the use of on-column or multimode inlet (MMI), but the LOD will be high regardless. Adamsite is analysed (LC-MS/MS) if its degradation product is found in the corresponding sample.

Proposed new target chemicals

New target degradation products of dumped phenylarsenic CWAs in marine environment are proposed in recent publications.[7,8] As the analytical methods for these chemicals are yet unvalidated, they are not included in this ROP, but should be considered in the future when analysing sediment samples from potentially contaminated areas.

Table 2. Names, CAS numbers, acronyms, and structures of the proposed new target chemicals.

Chemical CAS	Structure
10-methyl-5*H*-phenarsazin-10-oxide 21859-21-2	
Methyldiphenylarsine oxide 2887-09-4	

Sample Preparation

In most cases, the sediment samples will be received by the analytical laboratory frozen on dry ice. Blank sediment samples should be collected at sea at a distance from the dumping area. During sample preparation procedures, the sediment should not be dried and reconstituted in water since dried sediment oxidizes the analytes rapidly.

The basic principle of the sample preparation is to separate a sediment sample into two components: solid material (later called sediment portion) and water (later called pore water portion). The separation is done by centrifugation. The full sample preparation procedure is demonstrated in **Figure 1**.

The results for the sediment portion are given in µg/kg, where the weight of sediment is dry weight, dw. The dry weight of each sediment portion has to be determined separately, as the water contents of each sediment sample is different. The results for the pore water sample are given in µg/l of pore water.

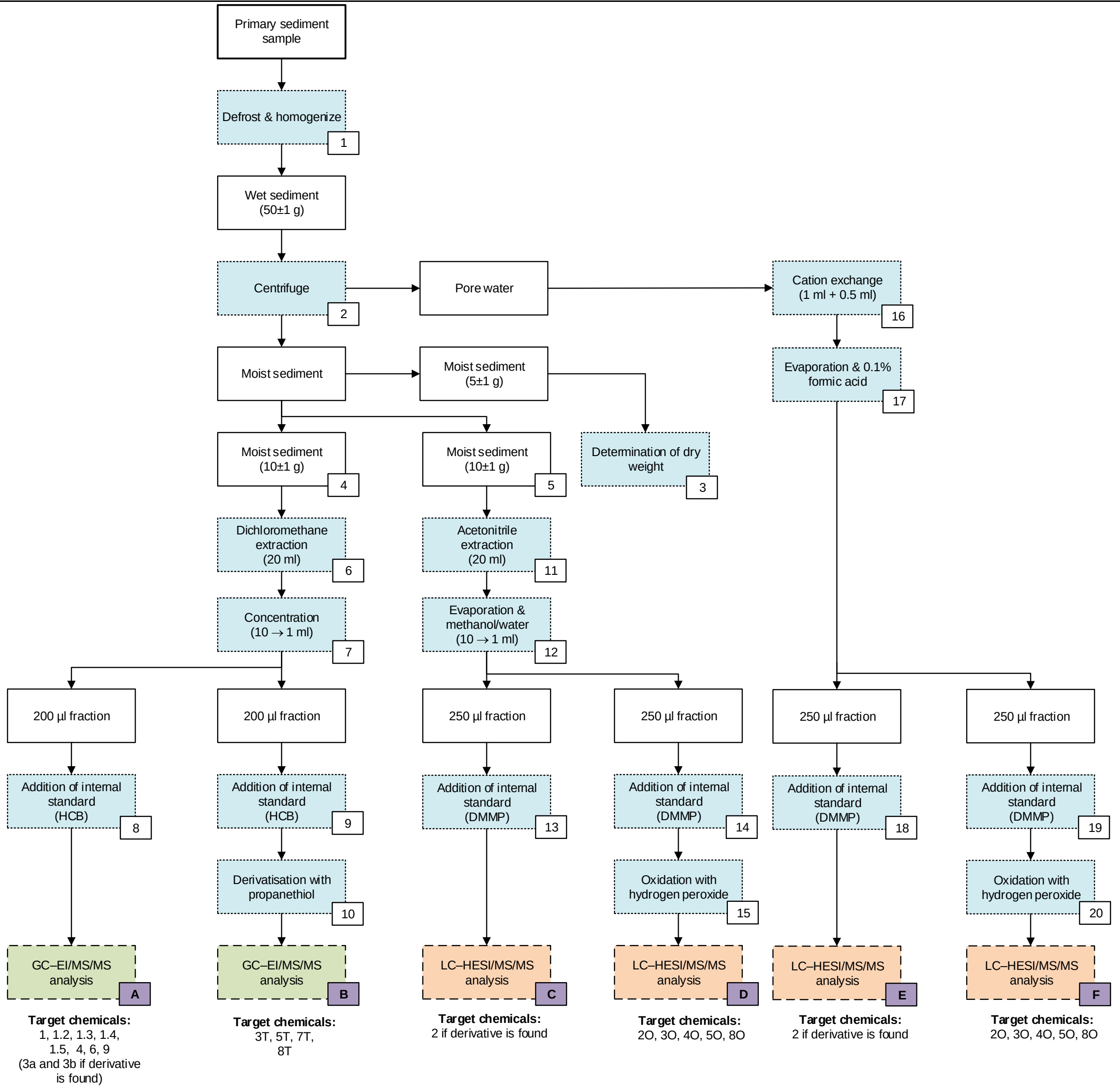


Figure 1. Sample preparation flow-chart for the sediment samples. Analyzed sample fractions are numbered **A** through **F**. The sample preparation steps 1 through 20 are described in detail in the text.

STEPS 1 and 2: Removal of Pore Water

A 50±1 g-portion of the sediment is weighed into a Falcon tube. The sediment is homogenized by using a plastic spoon before taking an aliquot. The remaining sediment should be stored in a freezer for possible later use. Next, the sample is centrifuged for 3 minutes at 1000 G, and excess pore water is collected and weighed.

STEP 3: Moisture Analysis

Moisture analysis is performed using Sartorius MA100H moisture analyzer (Göttingen, Germany) or similar. Standard drying program at 100 °C with semi-automatic ending (shut off parameter: maximum allowed weight change 1 mg/300 seconds) is used to determine the dry weight of the samples. A 5±1 g-portion of the centrifuged sediment is weighed on the sample pan and dried.

STEP 4 and 5: Weighing

Two 10±1 g-portions of the centrifuged sediment are weighed into separate Falcon tubes. The portions should consist of several small spoonfuls in order to represent the whole sediment sample.

STEP 6: Dichloromethane extraction

The first 10±1 g-portion of the centrifuged sediment is extracted with 10 ml of GC-MS grade dichloromethane by shaking for 10 minutes. After this, the sample is centrifuged for 3 minutes at 1000 G. The dichloromethane layer is decanted through filter paper (Whatman, 1PS or similar, rinsed with dichloromethane prior to use) into a 20 ml volumetric flask. The extraction is repeated with another 10 ml-portion of dichloromethane.

The dichloromethane layers from the two extractions are combined and adjusted with dichloromethane to 20 ml in a volumetric flask. The extract is transferred to a 20 ml EPA vial and dried with approximately 1 g of reagent grade anhydrous sodium sulphate. The DCM extract is left to stand overnight in the freezer.

STEP 7: Concentration

For the GC–MS/MS analysis, a 10 ml-portion of the dichloromethane extract is withdrawn with a 10 ml volumetric pipette, concentrated using TurboVap LV Evaporation System (Caliper Life Sciences, USA) or similar, and finally adjusted to a final volume of 1 ml in a volumetric flask. The sample is then filtrated using a Millex Samplicity 0.45 µm filter or similar.

STEPS 8 and 9: Addition of HCB as internal standard (ISTD)

Two 200 µl-portions of the concentrated dichloromethane extract are transferred into two vials (**8** and **9**) and 10 µl of internal standard (hexachlorobenzene, HCB, c = 1 µg/ml) is added to both.
The first fraction, **A,** will be analyzed by GC–MS/MS for **intact chemicals** (**1**, **1.2**, **1.3**, **1.4**, **1.5**, **4**, and **6.** For **3a** and **3b** only if derivative is found).

STEP 10: Derivatization with propanethiol (PrSH)

The second fraction is derivatized by adding 10 µl of propanethiol and incubating the sample at room temperature for 30 min.
This fraction, **B**, is analyzed by GC–MS/MS for **propanethiol derivatized chemicals** (**3T**, **5T**, **7T**, and **8T**.

STEP 11: Acetonitrile extraction

The second 10±1 g-portion of the centrifuged sediment is extracted with 10 ml of HPLC grade acetonitrile by shaking for 10 minutes, followed by centrifuging for 3 minutes at 1000 G. The acetonitrile layer is decanted through filter paper (Whatman 5 or similar) into a 20 ml volumetric flask.

The extraction is repeated with another 10 ml-portion of acetonitrile. The acetonitrile layers from the two extractions are combined and adjusted to a volume of 20 ml with acetonitrile in a volumetric flask. The extract is transferred to a 20 ml EPA vial and is stored in the freezer over night or until further sample preparation.

STEP 12: Solvent exchange

For the LC–MS/MS analysis, a 10 ml-portion of the acetonitrile extract is withdrawn with a 10 ml volumetric pipette and evaporated to dryness using GeneVac EZ-2 centrifugal evaporator or similar. The evaporation residue is reconstituted in 500 µl of LC-MS grade methanol and vortexed for 10 seconds followed by addition of 500 µl of UHQ water and vortexing for 10 seconds. The sample is filtered using a Millex Samplicity 0.2 µm filter or similar.

STEPS 13 and 14: Addition of DMMP as internal standard (ISTD)

Two 250 µl-portions of the concentrated acetonitrile extract are transferred into two vials and 15 µl of internal standard (dimethyl methylphosphonate, DMMP, c = 2 µg/ml) is added into both.
The first fraction, **C**, is intended for analysis by LC–MS/MS for **intact Adamsite (DM)** (**2**) if derivatives (**2O**) of the chemical are detected in **D**.

STEP 15: Derivatization with hydrogen peroxide (H_2O_2)
The second fraction is derivatized by adding 10 µl of 33 % hydrogen peroxide and incubating the sample at room temperature for 30 min.
This fraction, **D**, is analyzed by LC–MS/MS for **oxidized chemicals** (**2O**, **3O**, **4O**, **5O**, **8O**).

STEP 16: Cation exchange
Samples are prepared from the pore water collected in step 2. A 1 ml portion of the pore water sample is eluted through a cation exchange cartridge (CBA Bond elut, 100 mg/1 ml), which has been conditioned with 2 ml of methanol and 1 ml of water. After sample application, the cartridge is eluted with 0.5 ml of methanol. The eluent is combined with the flow through fraction to achieve a volume of approximately 1.5 ml.

STEP 17: Solvent exchange
The sample is evaporated to dryness using TurboVap LV Evaporation System (Caliper Life Sciences, USA) or similar, and the residue dissolved in 500 µl of 0.1% formic acid.

STEPS 18 and 19: Addition of DMMP as internal standard (ISTD)
Two 250 µl-portions of the cation exchanged sample are transferred into two vials and 15 µl of internal standard (dimethyl methylphosphonate, DMMP, c = 2 µg/ml) is added into both.
The first fraction, **E**, is intended for analysis by LC–MS/MS for **intact Adamsite (DM)** (**2**) if derivatives (**2O**) of the chemical are detected in **F**.

STEP 20: Derivatization with hydrogen peroxide (H_2O_2)
The second fraction is derivatized by adding 10 µl of 33% hydrogen peroxide and incubating the sample at room temperature for 30 min.
This fraction, **F**, is analyzed by LC–MS/MS for **oxidized chemicals** (**2O**, **3O**, **4O**, **5O**, **8O**).

Calculation of results

Several weighing steps are carried out during the sample preparation. The following section describes the methods for calculating the end results based on the weight of the samples. Figure 2 presents a flowchart of all weighted aliquots

Results in dry weight (µg/kg dw)
The dry weight of the sample (**m_{dw}**) is calculated by multiplying the weight of the used sample fraction (**m_5**) with the weight ratio of dry (**m_4**) and centrifuged wet sediment (**m_3**):

$$\boldsymbol{m_{dw} = m_5 \times \frac{m_4}{m_3}}$$

Calculation of the results per the original sample (R)
The results (**r**) from the instrument are obtained in pg/µl (ng/ml) based on the calibration curves. The unit of result of the calculation (**R**) is ng/g, which is the same as µg/kg.

*Intact sample for the GC–MS/MS analysis (**A**)*
The result (**r**) is first corrected for the dilution of the sample aliquot (originally 200 µl) due to the addition of the internal standard (10 µl): Correction factor is (200 µl + 10 µl)/200 µl = 1.05.

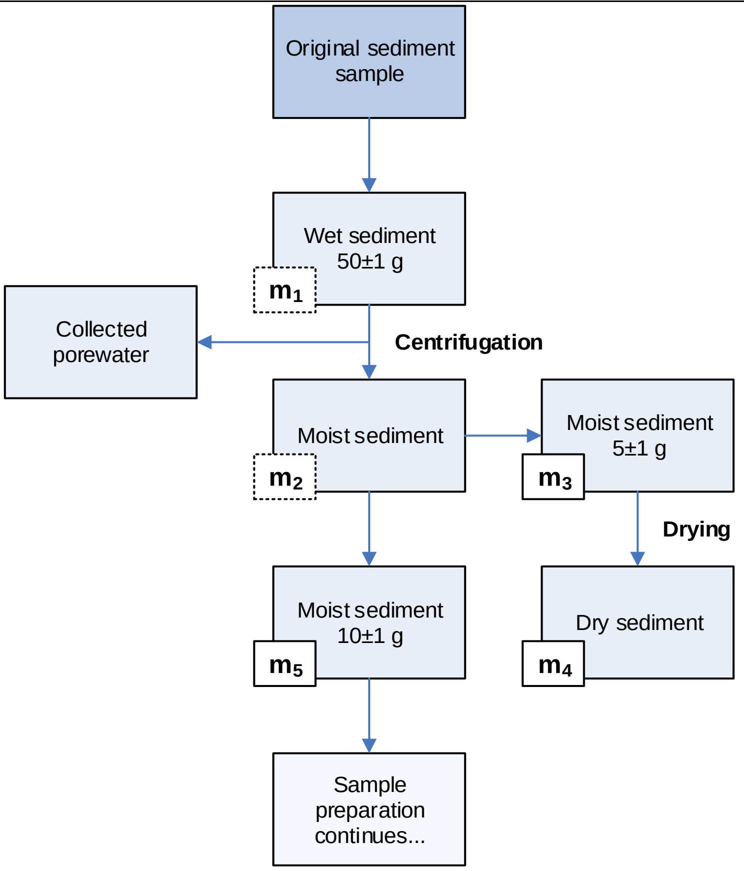


Figure 2. Flowchart describing the weighing process.

Then the result is corrected for the concentration of the dichloromethane extract: Correction factor is 0.1.

Finally, the concentration of the target chemical in the dichloromethane is converted into actual amount in the extract by multiplying by the volume (20 ml).

The formula used for fraction **A** is:

$$\boldsymbol{R} = \frac{\boldsymbol{r} \times 1.05 \times 0.1 \times 20\ ml}{\boldsymbol{m_{dw}}}$$

*Derivatized sample for the GC–MS/MS analysis (**B**)*

The result (**r**) is first corrected for the dilution of the sample aliquot (originally 200 µl) due to the addition of derivatization reagent (10 µl) and the internal standard (10 µl):

Correction factor is (200 µl + 10 µ + 10 µl)/200 µl = 1.10.

Then the result is corrected for the concentration of the dichloromethane extract: Correction factor is 0.1.

Finally the concentration of the target chemical in the dichloromethane is converted into actual amount in the extract by multiplying by the volume (20 ml).

The formula used for fraction **B** is:

$$\boldsymbol{R} = \frac{\boldsymbol{r} \times 1.10 \times 0.1 \times 20\ ml}{\boldsymbol{m_{dw}}}$$

*Intact sample for the LC–MS/MS analysis (**C**)*

The result (**r**) is first corrected for the dilution of the sample aliquot (originally 250 µl) due to the addition of the internal standard (15 µl):

Correction factor is (250 µl + 15 µl)/250 µl = 1.06.

The concentration of the sample is not changing in the solvent exchange process.

Then the result is corrected for the concentration of the acetonitrile extract: Correction factor is 0.1.

Finally the concentration of the target chemical in the acetonitrile is converted into actual amount in the extract by multiplying by the volume (20 ml).

The formula used for fraction **C** is:

$$\boldsymbol{R} = \frac{\boldsymbol{r} \times 1.06 \times 0.1 \times 20\,ml}{\boldsymbol{m_{dw}}}$$

*Derivatized sample for the LC–MS/MS analysis (**D**)*

The result (**r**) is first corrected for the dilution of the sample aliquot (originally 250 µl) due to the addition of oxidation reagent (10 µl) and the internal standard (15 µl): Correction factor is (250 µl + 15 µl + 10 µl)/250 µl = 1.10.

The concentration of the sample is not changing in the solvent exchange process.

Then the result is corrected for the concentration of the acetonitrile extract: Correction factor is 0.1.

Finally the concentration of the target chemical in the acetonitrile is converted into actual amount in the extract by multiplying by the volume (20 ml).

The formula used for fraction **D** is:

$$\boldsymbol{R} = \frac{\boldsymbol{r} \times 1.10 \times 0.1 \times 20\,ml}{\boldsymbol{m_{dw}}}$$

*Derivatized sample for the LC–MS/MS analysis (**F**)*

The result is first corrected for the dilution of the sample aliquot (originally 250 µl) due to the addition of oxidation reagent (10 µl) and the internal standard (15 µl): Correction factor is (250 µl + 15 µl + 10 µl)/250 µl = 1.10.

Then the result is corrected for the concentration of the sample: Correction factor is 0.5.

Derivatization reactions

The reactive warfare agents degrade in aqueous conditions at variable rates. Additionally, time of their release from munitions or containers is unknown. Thus, it can be expected that the chemicals are at least partially degraded. Natural degradation of the warfare agents proceeds normally via hydrolysis and/or oxidation. Figure 3 – Figure7 show the most probable degradation products and routes for Adamsite (DM) (**2**), Clark I (DA) (**3a**), Clark II (DC) (**3b**), Lewisite I (L1) (**7**), Lewisite II (L2) (**8**), and phenyldichloroarsine (PDCA) (**5**).

The applied main derivatization methods, oxidation with hydrogen peroxide and derivatization with propanethiol, derivatize both the intact warfare agents and their degradation products. Therefore, the results obtained using derivatization are always the sum of all related chemicals.

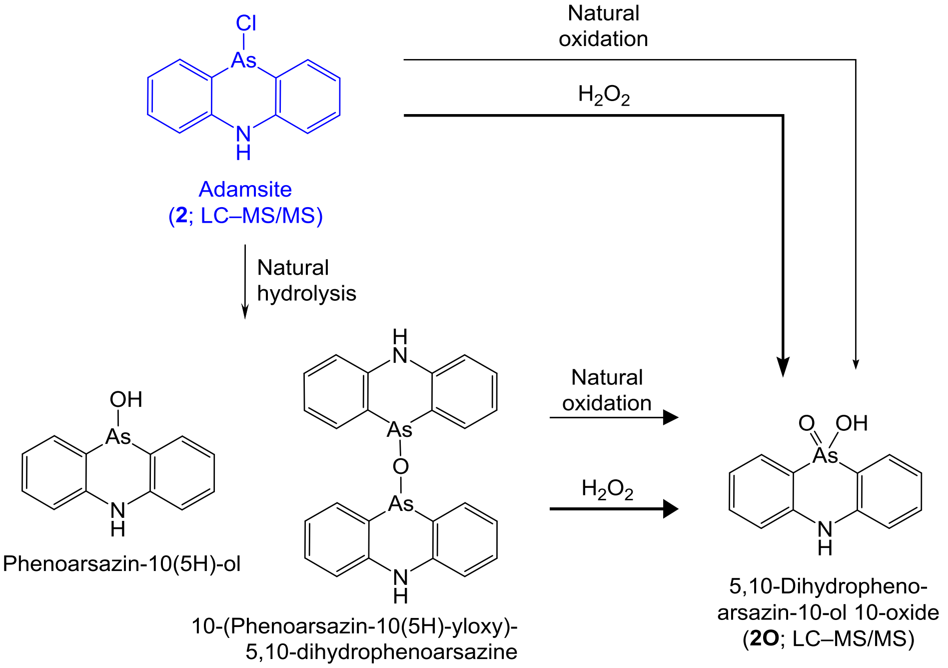


Figure 3. Derivatization reactions of Adamsite (DM) (**2**) and its degradation products using hydrogen peroxide (for LC–MS/MS) to form **2O**.

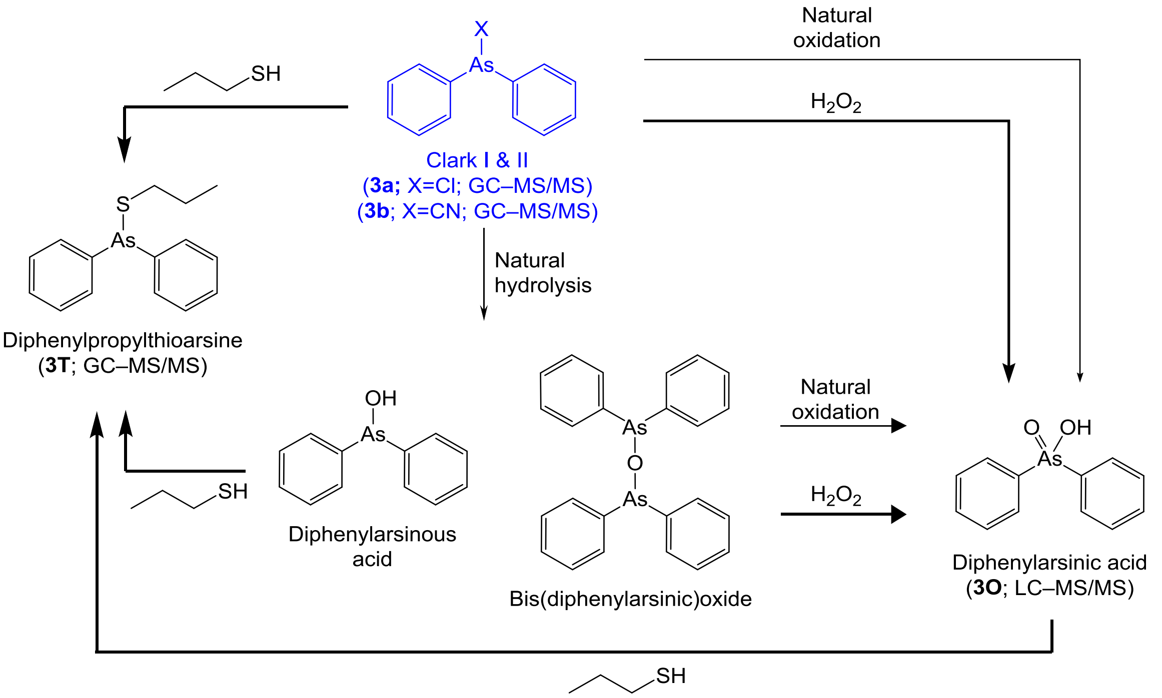


Figure 4. Derivatization reactions of Clark I (DA) (**3a**), Clark II (DC) (**3b**) and their degradation products using hydrogen peroxide (LC–MS/MS) to form diphenylarsinic acid (**3O**) or using propanethiol (for GC–MS/MS) to form diphenylpropylthioarsine (**3T**).

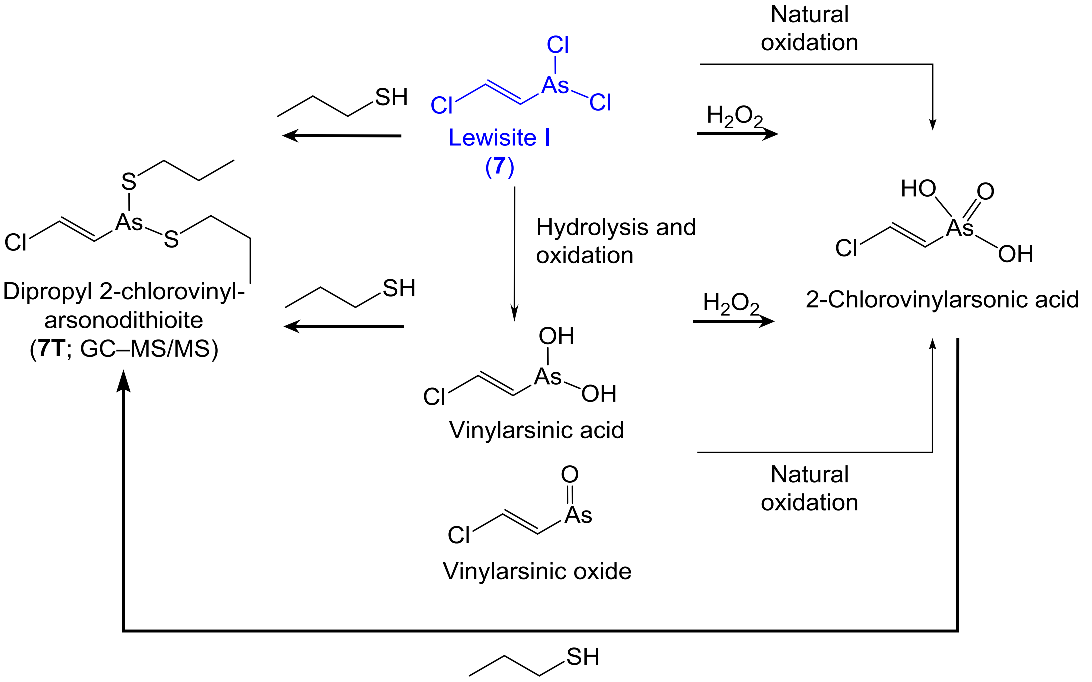


Figure 5. Derivatization reactions of Lewisite I (L1) (**7**) and its degradation products using propanethiol (for GC–MS/MS) to form dipropyl 2-chlorovinylarsonodithioite (**7T**).

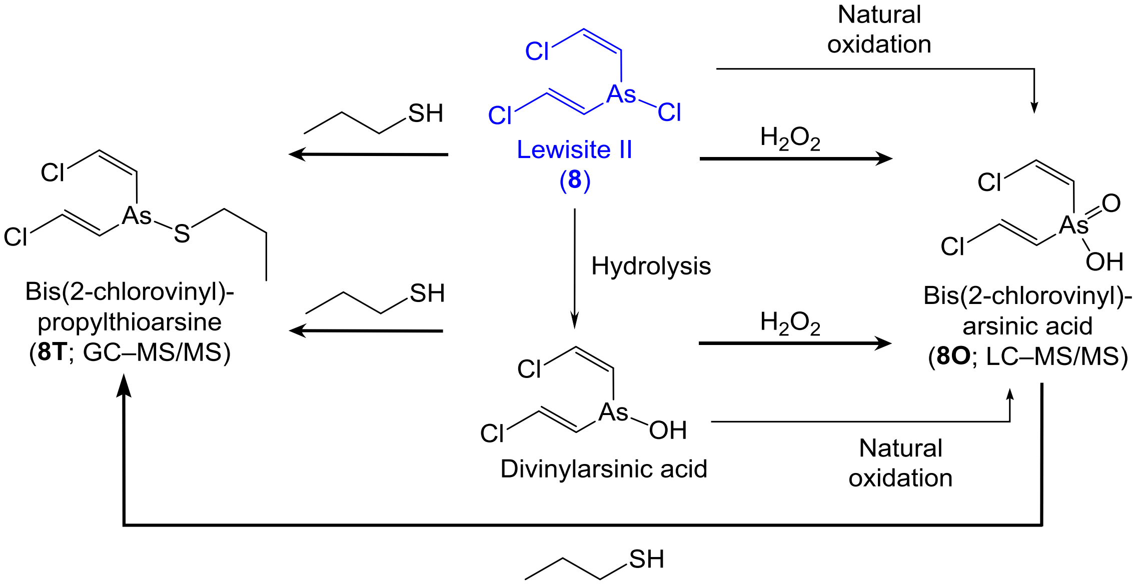


Figure 6. Derivatization reactions of Lewisite II (L2) (**8**) and its degradation products using hydrogen peroxide (for LC–MS/MS) as bis(2-chlorovinyl)arsenic acid (**8O**) or using propanethiol (for GC–MS/MS) to form bis(2-chlorovinyl)propylthioarsine (**8T**).

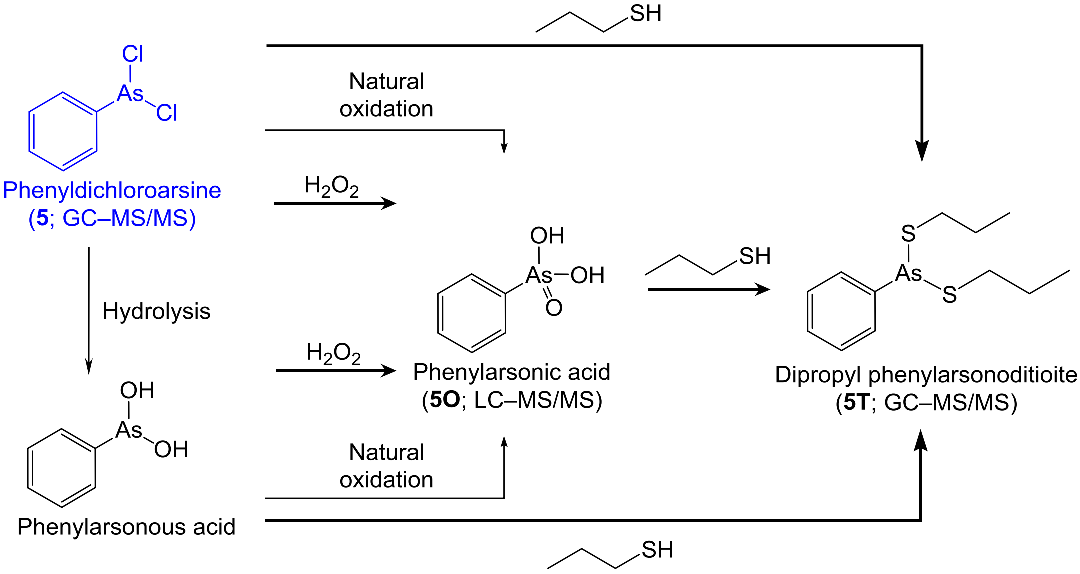


Figure 7. Derivatization reaction of phenyldichloroarsine (PDCA) (5) and its degradation products using propanethiol (for GC–MS/MS) to form dipropyl phenylarsonodithioite (5T) or using hydrogen peroxide (for LC–MS/MS) to form phenylarsonic acid (5O).

Preparation of calibration standards

The calibration standards are prepared in extracts of blank sediment. The reference chemicals are dissolved in acetone. Dilutions for spiking are made in acetone or dichloromethane. As in the case of prepared samples, the internal standard (ISTD) used is hexachlorobenzene (HCB) for GC–MS/MS and dimethyl methylphosphonate (DMMP) for LC–MS/MS. All standards are made by spiking the chemicals into blank sediment extracts, which has been identically prepared.

For GC-MS/MS technique, a six to eight-point calibration curve (ca. 0.5–100 pg/µl, depending on expected sample concentrations) is applied for intact compounds, and a six to eight-point calibration curve (ca. 1–200 pg/µl, depending on the expected sample concentrations) is applied for derivatized samples.

For LC–MS/MS an eight point calibration curve (ca. 1–150 pg/µl) is applied for oxidized samples. When the oxidation product of Adamsite (2O) is found from the samples, a calibration curve is applied to the intact LC-MS/MS samples (see Table 1).

Mass spectrometric methods

Gas chromatography–tandem mass spectrometric (GC–MS/MS) method

Both GC–MS/MS fractions (**A** and **B**) are analyzed using the following analysis method or similar. This standard operation procedure describes analysis method using an Agilent Technologies 7890A gas chromatograph (GC) equipped with an automatic liquid injector and an Agilent Technologies 7010 GC/MS Triple Quadrupole mass spectrometer. Capillary column is a DB-5MS UI (Agilent, 30 m x 0.25 mm i.d., 0.25 µm film). The column temperature is programmed from 40 °C (isothermal time 1 min) to 290 °C at 10 °C/min and held at final temperature for 10 min. Splitless injection (splitless time 1 min) is used with the injector temperature of 250 °C. Injection volume is 1-2 µl. The carrier gas is helium with a flow of 35 cm/sec at 40 °C. The transfer line temperature between the GC and MSD is 290 °C.

The analysis is performed using electron ionization technique followed by multiple reaction monitoring (MRM) (see Table 3 for conditions) with 3 selective transitions. The described method can be translated to any corresponding GC-MS/MS analytical system.

Table 3. Examples of the MRM conditions used for the GC–MS/MS analysis.

#	Chemical (acronym)	Reactions (*m/z*) and collision energies (eV) (in brackets)		
		Quantifier Q	**Qualifiers q**	
Intact chemicals				
1	Sulfur mustard (H)	109→63 (7)	109→73 (7)	160→109 (7)
1.2	1,4-Dithiane	120→61 (12)	120→46 (40)	92→46 (7)
1.3	1,4-Oxathiane	104→61 (7)	104→46 (25)	74→46 (7)
1.4	1,4,5-Oxadithiepane	136→64 (25)	136→92 (7)	89→35 (25)
1.5	1,2,5-Trithiepane	152→87 (7)	152→92 (7)	124→60 (12)
6	α-Chloroacetophenone (CN)	154→105 (0)	105→77 (12)	105→51 (40)
4	Triphenylarsine (TPA)	152→77 (25)	306→152 (7)	152→51 (40)
9	Tabun	133→106 (7)	162→70 (0)	-
–	Hexachlorobenzene (ISTD)	284→249 (25)	284→214 (40)	284→142 (50)
Propanethiol derivatives				
3T	Diphenylpropylthioarsine	261→183 (15)	304→261 (7)	183→107 (17)
5T	Dipropyl phenylarsonodithioite	302→259 (5)	302→227 (7)	227→153 (10)
7T	Dipropyl (2-chlorovinyl)-arsonodithioite	286→176 (5)	286→43 (22)	243→165 (10)
8T	Bis(2-chlorovinyl) propylthioarsine	272→127 (7)	229→203 (5)	203→167 (5)
–	Hexachlorobenzene (ISTD)	284→249 (25)	284→214 (35)	284→142 (50)
Intact Clark I and Clark II				
3a	Clark I	154→153 (20)	264→154 (12)	154→152 (35)
3b	Clark II	154→152 (35)	255→154 (2)	154→153 (12)

Criteria for identification

In the methods described here, three MRM-transitions are used. One quantifier-qualifier pair would be acceptable, but with two pairs, a higher confidence level of the identification can be reached.

To evaluate the validity of the identifications of each chemical in the samples, identification criteria specified by the EU are followed.[9] Identification of a chemical should not be accepted if it does not fulfil the criteria.

The retention time of the peak in the sample must fall within a window of ± 0.1 min of the retention time of the reference chemical. Relative ion intensities (ion ratios) are determined according to the instructions [§] using the following tolerances summarized in Table 4.

Table 4. EU criteria for ion/reaction ratios.[9]

Relative intensity (% of base peak)	Allowed relative tolerance
> 50 %	± 20 %
> 20 to 50 %	± 25 %
> 10 to 20 %	± 30 %
≤ 10 %	± 50 %

§ “The relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense ion or transition, shall correspond to those of the calibration standard, either from calibration standard solutions or from spiked samples, at comparable concentrations, measured under the same conditions.”

Practical use of the identification criteria are illustrated in Figure 4 presenting GC–MS/MS data from a sediment sample from the Baltic Sea. The retention time difference between the reference sample (**R**) and the actual sample (**S**) is within the allowed limit ±0.1 min. The ratios of the two qualifier reactions (152→92 and 124→60) compared to the quantifier reaction (152→87) were 47.6 % and 25.7 %, respectively. The allowed limits for these ratios were 47.3±11.8 % and 26.3±6.6 %, respectively. Based on these two criteria the example identification in Figure 4 can be accepted.

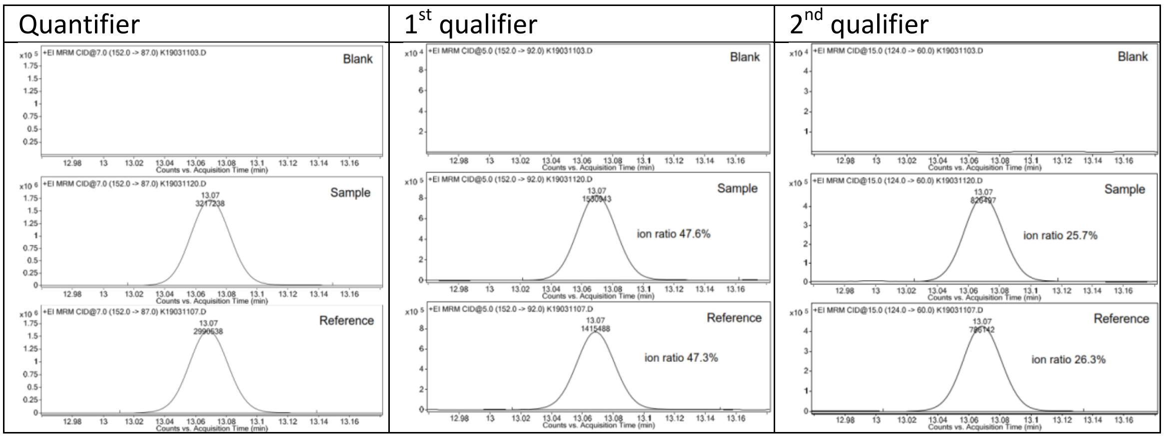


Figure 8. Example use of identification criteria in identification of mustard degradation product 1,2,5-trithiepane (**1.5**) using GC–EI/MS/MS technique. Presented MRM chromatograms are for blank sediment sample, contaminated sediment sample, and a 5 ppb reference sample.

Quality control

The performance of the instrument should be tested daily (at the beginning and at the end of each analysis batch) following quality control routines. For GC–MS/MS chemical warfare agent analysis the recommended quality control is carried out with standard sample consisting of the following chemicals (5 ng/µl):

- trimethylphosphate
- 2,6-dimethylphenol
- 5-chloro-2-methylaniline
- tri-n-butylphosphate
- dibenzothiophene
- malathion
- methyl stearate
- *n*-alkanes, even members (C_8–C_{24})

The control sample used for the analysis is a standard solution containing all monitored chemicals at the concentration levels of ca. 1 pg/µl and 10 pg/µl for intact chemicals and ca. 25 pg/µl for propanethiol derivatives. The additional lower level (1 pg/µl) control sample for the intact chemicals is included for accuracy of the calibration, since many degradation products for sulfur mustard (H) (**1**) have been present at concentrations close to this level in previous studies. Both control samples are analyzed once or twice during the sample batch, depending on the total number of samples. Examples of control sample runs are presented in Figure 9 (for intact target chemicals) and Figure 10 (for derivatized target chemicals).

Blank samples (both solvent and sediment) should be analyzed to examine the possibility of contamination arising from sample preparation or from instrumental analysis. Three types of blanks should be included in the sample batches: solvent blank (no sample preparation), sediment blank (similarly prepared known blank sediment), quality blank (similar sample preparation without sediment).

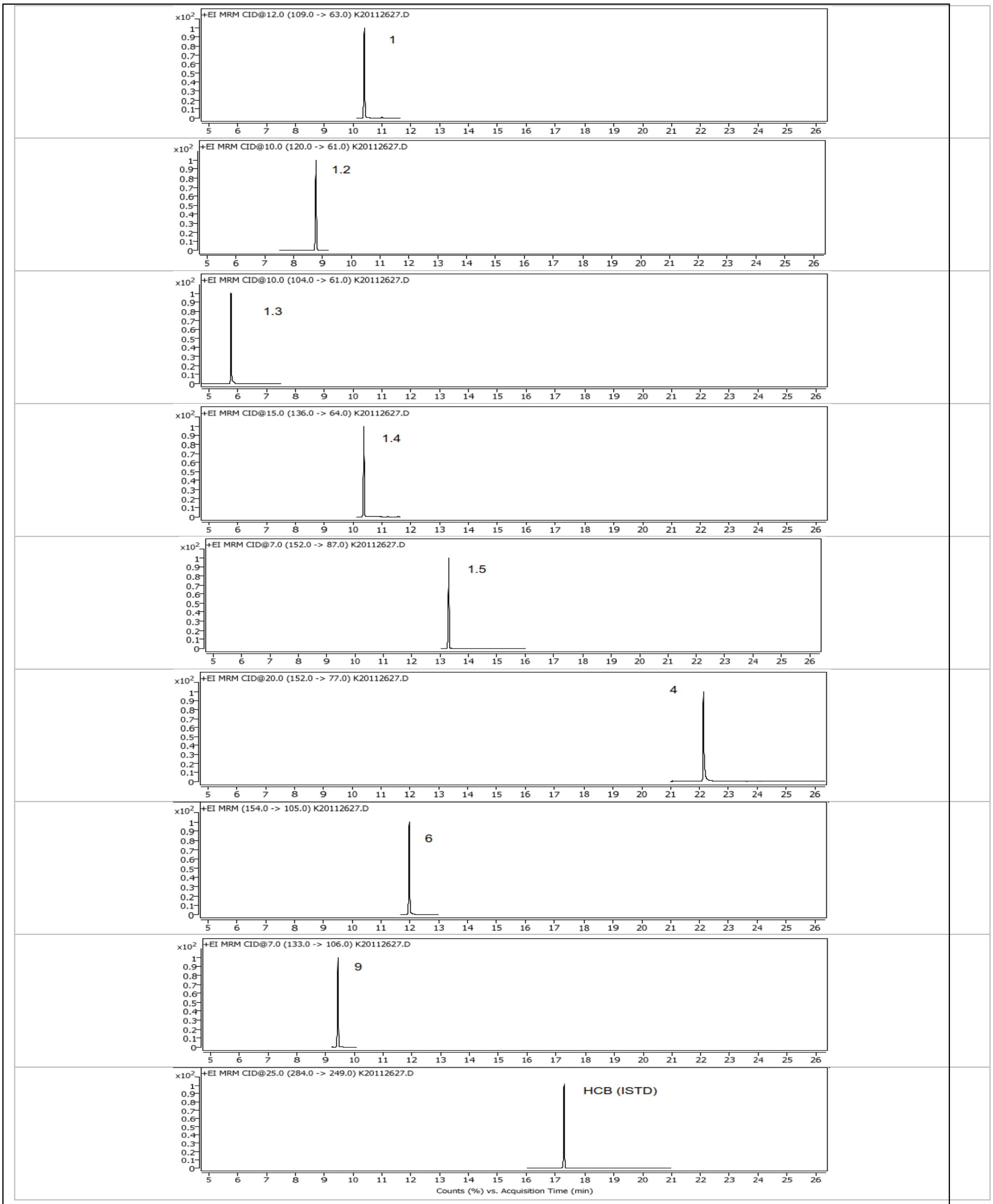


Figure 8. GC–EI/MS/MS MRM chromatograms for ca. 10 pg/µl standards of intact chemicals.

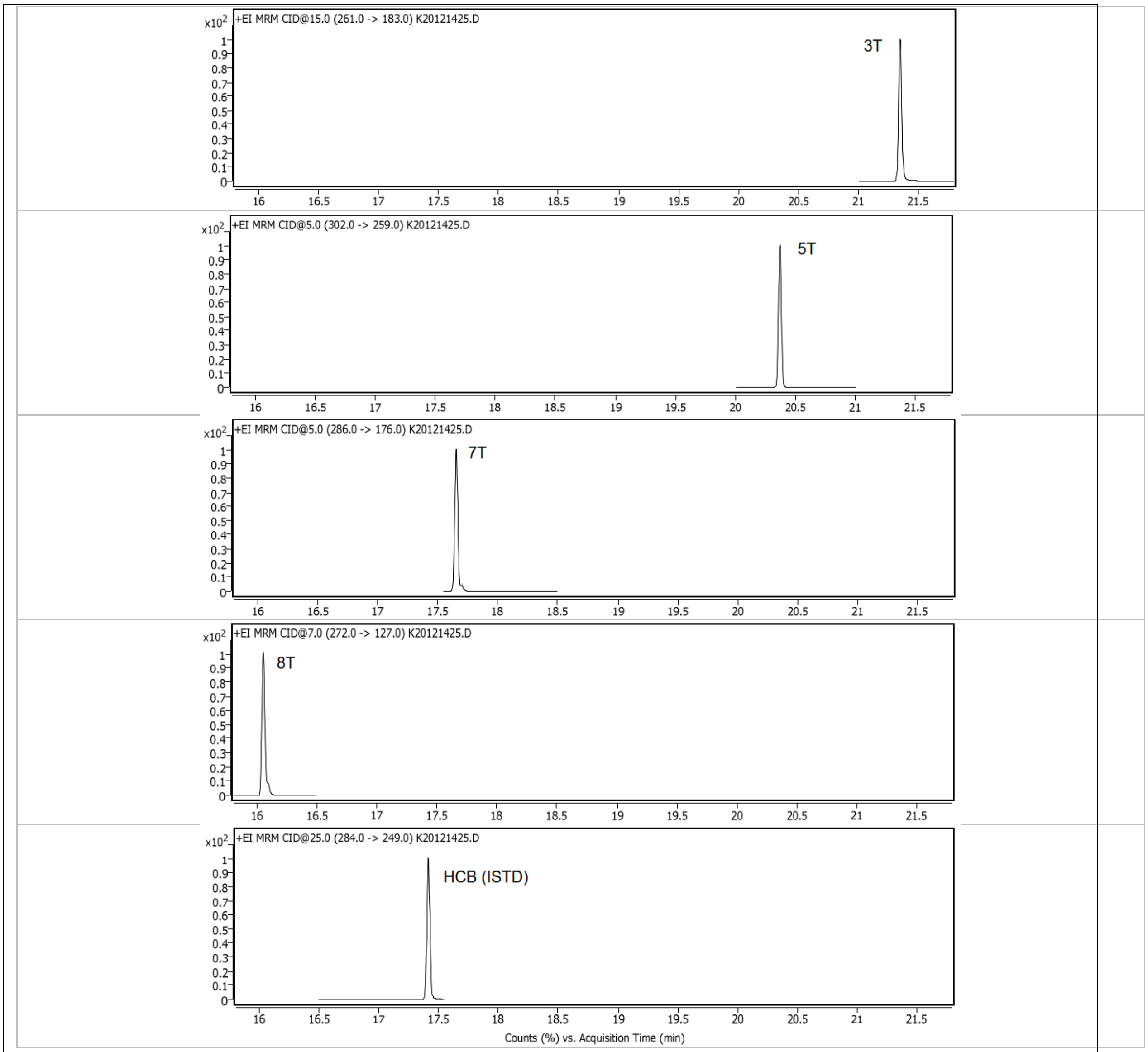


Figure 9. GC–EI/MS/MS MRM chromatograms for ca. 25 pg/µl standards of propanethiol derivatives.

Liquid chromatography–tandem mass spectrometric (LC–MS/MS) method

All LC–MS/MS fractions (C - F) are analyzed using a Shimadzu Nexera liquid chromatograph and Thermo Scientific TSQ Quantum Ultra triple quadrupole mass spectrometer. The analysis is carried out using heated electrospray ionization (HESI) technique followed by multiple reaction monitoring (MRM) (see Table 5 for conditions). All compounds are analyzed in positive ion mode.

The described method can be translated to any corresponding LC-MS/MS analytical system.

Table 5. The MRM conditions used for the LC–MS/MS analysis.

#	**Chemical**	**Reaction (*m/z*)**		**Collision energy (eV)**	**Ionization**
		Quantifier Q	**Qualifier q**		
2	Adamsite (DM)	242 → 167	242 → 139	30/45	HESI POS
2O	5,10-Dihydrophenarsazin-10-ol 10-oxide	276 → 230	276 → 154	20	HESI POS
3O	Diphenylarsinic acid	263 → 141	263 → 152	25	HESI POS
4O	Triphenylarsine oxide	323 → 227	323 → 154	38	HESI POS
5O	Phenylarsonic acid	203 → 77	203 → 109	25/18	HESI POS
8O	Bis(2-chlorovinyl)arsinic acid	231 → 145	231 → 159	23	HESI POS
–	Dimethyl methylphosphonate (ISTD)	125 → 93	125 → 111	20	HESI POS

The liquid chromatographic separation is done using a gradient elution and a Waters XBridge BEH C18 column (2.1 x 100 mm, particle size 2.5 µm) with an XBridge BEH C18 VanGuard Cartridge pre-column (2.1 x 5 mm, particle size 2.5 µm). The column temperature is 30 °C and the injection volume is 5 µl. The gradient with a flow of 0.4 ml/min is created using two solvent mixtures: solvent A: water containing 0.1% formic acid and solvent B: methanol containing 0.1% formic acid. For the oxidized samples the amount of solvent B is increased from 5% at 0 min to 100% at 3 min. After this, the B is kept at 100% for 1 min and then at 5% for 1 min. For the intact samples solvent B is increased from 5% at 0 min to 100% at 2 min.

Criteria for identification

To evaluate the validity of the identifications of each chemical in the samples, identification criteria specified by the EU are followed.[9] Identification of a chemical should not be accepted if it does not fulfil the criteria. The retention time of the peak in the sample must fall within a window of ±0.2 min of the retention time from the reference chemical. Relative ion intensities (ion ratios) are determined according to the instructions using the tolerances summarized in Table 4.

Quality control

The performance of the instrument is tested daily following quality control routines. For LC–MS/MS chemical warfare agent analysis the quality control is carried out with a control sample consisting of the following chemicals (10 ng/µl each)

- Butyl dihydrogen phosphate
- Butylphosphonic acid
- Methionine
- 2-(N-methyl-N-propylamino)ethanol
- Dibutyl hydrogen phosphate
- Tributyl phosphate

A standard containing all monitored chemicals at the concentration level of ca. 50 pg/µl is analyzed once or twice during each sample batch, depending on the number of samples. Blanks are analyzed to examine the possibility of contamination arising from sample preparation or analysis. Three types of blanks should be included in the sample batches: solvent blank (no sample preparation), sediment blank (similarly prepared known blank sediment), quality blank (similar sample preparation without sediment).

Examples of total ion chromatograms for a ca. 50 pg/µl (oxidized) and ca. 500 pg/µl (intact) standard samples are presented in Figure 11 and Figure 12.

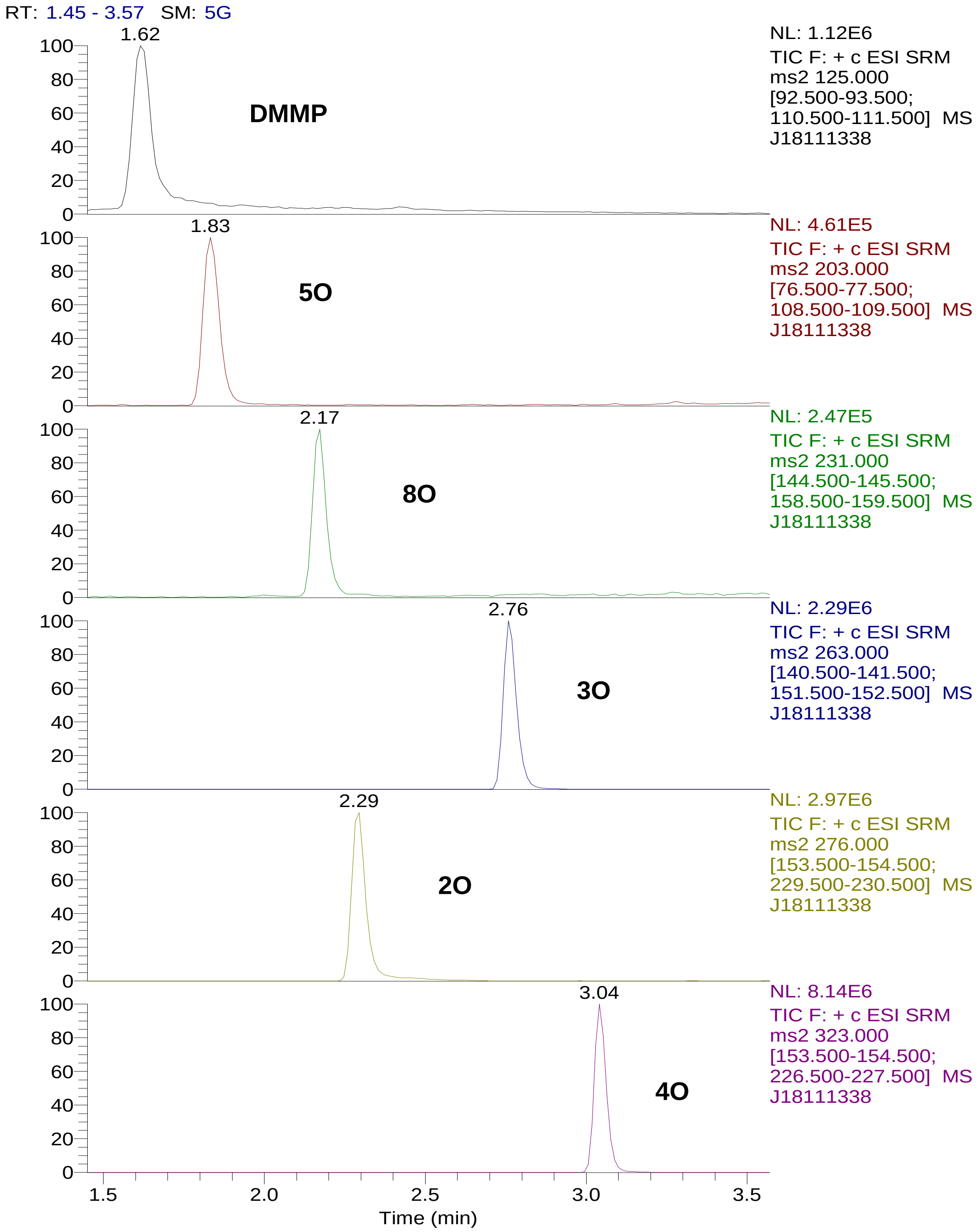


Figure 10. Example LC–HESI/MS/MS MRM chromatograms for 50 pg/µl standards (corresponding to ca. 20 µg/kg dw in sediment) for **oxidized chemicals**.

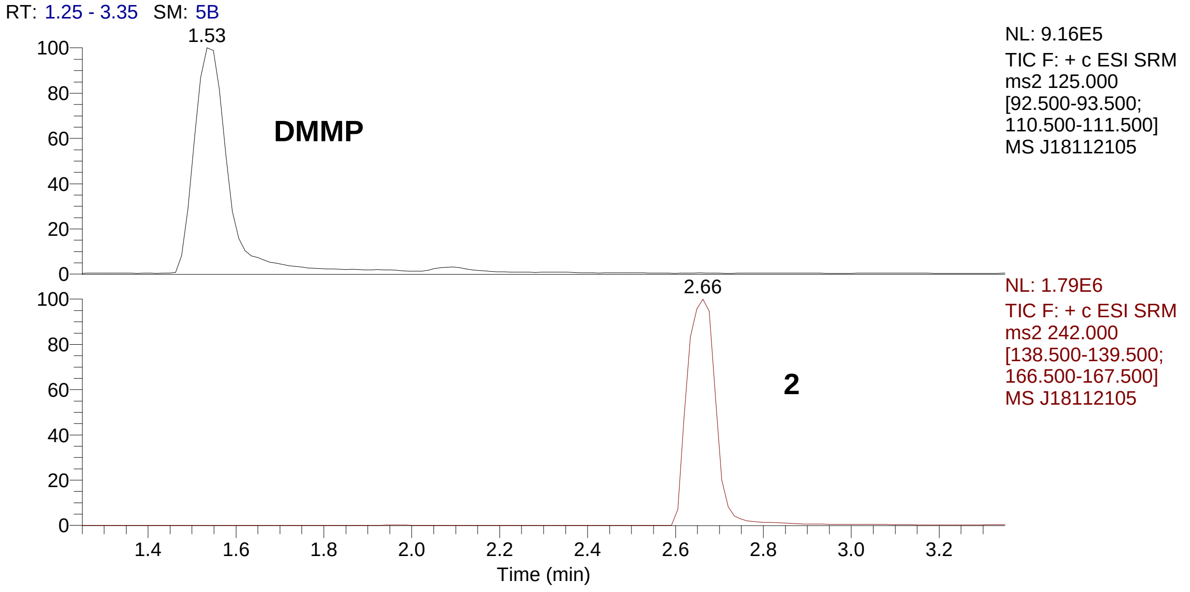


Figure 11. Example LC–HESI/MS/MS MRM chromatograms for ca. 500 pg/µl standard (corresponding to ca. 190 µg/kg dw in sediment) for **intact chemicals**.

Validation

The analysis data should be validated per analyzing laboratory's established quality and validation procedures. At the VERIFIN laboratory, the validation procedures and criteria of the Institute are followed for the GC-MS/MS and LC-MS/MS methods. The lower limit of quantitation (LLOQ) values for GC-MS/MS range from 0.5 to 1 ppb for intact chemicals and from 1 to 2.5 ppb for derivatized chemicals. For LC-MS/MS, the LLOQ values range from 3 to 5 ppb for oxidized chemicals and is ~15 ppb for intact Adamsite.

Conclusions (if applicable)

This ROP describes sample preparation and analytical methods for analysis of CWA related chemicals in sediment and pore water samples utilizing GC-MS/MS and LC-MS/MS techniques. The LLOQ values for GC-based methods are generally from 0.5 to 1 ppb for intact chemicals and from 1 to 2.5 ppb for derivatized chemicals. For LC-based mehtods, the LLOQ values range from 3 to 5 ppb for oxidized chemicals and is ~15 ppb for intact Adamsite.

References

[1] CHEMSEA project website: http://www.chemsea.eu/

[2] Söderström, WP3: Summary of chemical analysis of sediment samples, Report of the CHEMSEA Project, 2.9.2014, 59 pages.

[3] Nyholm *et al.*, Analysis of chemical warfare related compounds in sediment samples from the Baltic Sea performed at the Swedish Defence Research Institute in the CHEMSEA project, Report of the CHEMSEA Project, 26.11.2013, 45 pages.

[4] Popiel *et al.*, Chemical analysis of sediment and core samples performed at Military University of Technology (MUT) , Report of the CHEMSEA Project, 20.12.2013, 47 pages.

[5] Söderström *et al.*, Chemical analysis of sediment samples performed at Finnish Institute for Verification of the Chemical Weapons Convention (VERIFIN), Report of the CHEMSEA Project, 3.2.2014, 51 pages.

[6] Söderström *et al*., Analysis of sediment samples for sea-dumped chemical weapons, Recommended Operating Procedures for Analysis in the Verification of Chemical Disarmament, Blue Book, 2017 Edition, The Ministry for Foreign Affairs of Finland, University of Helsinki, ISBN 978-951-51-3916-0 (paperback), ISBN 978-951-51-3917-7 (PDF), Section 3, Part F, Chapter VIII (http://www.helsinki.fi/verifin/bluebook)

[7] Niemikoski *et al*. Identification of Degradation Products of Sea-Dumped Chemical Warfare Agent-Related Phenylarsenic Chemicals in Marine Sediment, Anal. Chem, 2020

[8] Niemikoski *et al*. Studying the metabolism of toxic chemical warfare agent-related phenylarsenic chemicals in vitro in cod liver, *J. Haz. Mat*, 2020

[9] Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC), Official Journal of the European Communities, 2002, 45 (L221) 8–36.

Change history

1.0	Date	First edition
1.1	18.5.2021	Definition of the document was changed from SOP to ROP.

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