Recommended Operating Procedure (ROP)

Aim of ROP

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

31. Chemical analysis of degradation products of phenylarsenic CWAs in fish

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Scope

This Recommended Operation Procedure (ROP) describes analysis of selected degradation products of sea-dumped phenylarsenic chemical warfare agents (CWAs) in fish samples. The ROP includes sample preparation, analytical methods, and the evaluation of the produced data. This ROP is also applicable for other marine biota samples, such as lobsters and shrimps.

Summary of the ROP

This ROP describes a chemical analysis method to study the exposure of fish to CWA-related phenylarsenic chemicals, and the possible accumulation of these chemicals in the fish. This ROP includes sample pre-treatment methods for fish cut fillet and liver samples, instrument analysis methods and interpretation of the produced analytical data. This ROP is based on the method published by Niemikoski *et al*. 2020.[1]

The list of target chemicals of this ROP is based on findings from sampling campaigns of DAIMON project where traces of CWA-related phenylarsenic chemicals have been detected from different marine biota samples.

Chemical analysis of CWA-related phenylarsenic chemicals in fish is based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) in multiple reaction monitoring (MRM) mode. Identification criteria for the detection of chemicals are based on European Union guidelines.[2]

This method is suitable when screening target chemicals at trace levels (> 0.2 ng/g) in fish samples.

Safety aspects

General safety aspects, when conducting laboratory work, should be followed when handling any samples or chemicals. Safety data sheet (SDS) of phenylarsenic chemicals must be read carefully. Pay attention to the instructions for the disposal of toxic arsenic waste. Biota samples should be treated as any other samples containing hazardous chemicals according to laboratory's safety instructions.

Documentation

When samples are received in the laboratory, each fish 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.

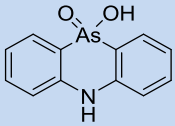
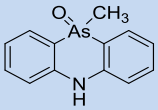
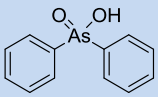
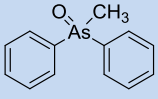
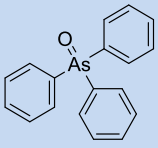
Methods

TARGET CHEMICALS

Quantitative analysis of target chemicals is needed to prove the presence of degradation products of dumped phenylarsenic CWAs in marine biota and to support the risk assessment for possible accumulation in the food chain.

Arsenic containing CWAs: Adamsite, Clark I and II, triphenylarsine, are hydrolysed and/or oxidized in aquatic environment forming oxidized products of these agents. Moreover, Adamsite and Clark I are known to form methylated degradation products both in marine sediment and as the result of biotransformation reactions in fish.[3,4] Therefore, the oxidized and methylated forms of these chemicals have been selected as target chemicals. Table 1 summarizes the names of the studied chemicals, their CAS numbers, and structures.

Table 1. Chemical names, CAS numbers, and structures of the studied chemicals.

Chemical CAS	Structure
5,10-Dihydrophenarsazin-10-ol 10-oxide 4733-19-1	
10-methyl-5*H*-phen-arsazin-10-oxide 21859-21-2	
Diphenylarsinic acid 4656-80-8	
Methyldiphenylarsine oxide 2887-09-4	
Triphenylarsine oxide 1153-05-5	

SAMPLE PREPARATION

FISH MUSCLE:

STEP 1: Homogenization
In general, a 5 ± 0.5 g portion of the fish cut fillets are homogenized using tissue homogenizer. Each sample should have its own container for homogenization to prevent possible cross-contamination. In case the water content of the sample is very low, 0.8-1.0 ml UHQ water can be added to ensure proper homogenization of the sample.

STEP 2: Extraction with acetronitrile
1.5 mL of hydrogen peroxide (33 %) is added to the homogenized tissue sample prior to extraction with 20 mL acetonitrile (ACN). The samples are shaken vigorously for 15 minutes, and then a small amount of sodium chloride (ca. 0.5 g) is added followed by centrifugation for 4 minutes at 5000 rpm. After centrifugation, the ACN phase is separated and evaporated to dryness.

STEP 3: Solid phase extraction and solvent exchange
The samples are reconstituted in 1 mL UHQ water and shaken vigorously for 1 min. Next, the samples are loaded onto reversed-phase (C18) solid phase extraction (SPE) cartridges (e.g. Agilent Bond Elut C18). The cartridges are conditioned with 1 mL methanol followed by 1 mL UHQ water prior to sample loading. The cartridges are washed with 450 µL of 5 % methanol, and the analytes are eluted with 2 x 450 µL of 1 % formic acid in methanol. The samples are evaporated to dryness and reconstituted in 400 µL methanol:water 1:1 (v/v). The samples are filtrated with 0.20 µm filter prior instrument analysis.

The full sample preparation procedure is demonstrated in Figure 1.

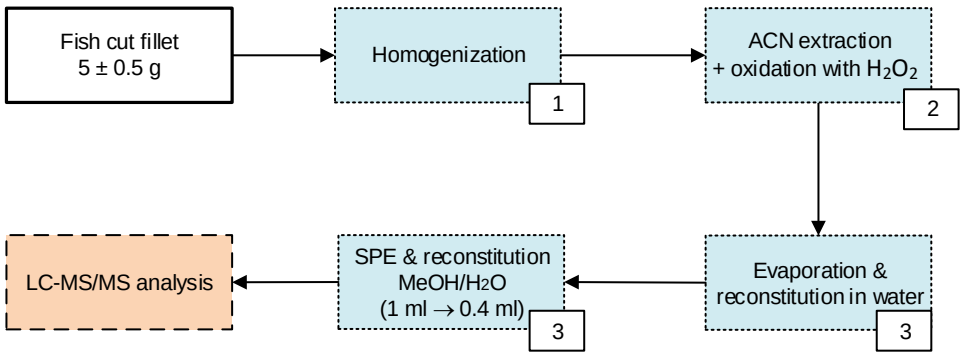


Figure 1. Sample flow chart for fish cut fillet samples

FISH LIVER
STEP 1: Acetonitrile homogenization
In general a 3 ± 0.3 g portion of the fish liver is homogenized using tissue homogenizer. Each sample should have its own container for homogenization to prevent possible cross-contamination.

STEP 2: Acetonitrile extraction and wash
1.5 mL of hydrogen peroxide (33 %) is added to the homogenized tissue samples prior to extraction with 20 mL acetonitrile (ACN). The samples are shaken vigorously for 15 minutes, and then a small amount of sodium chloride (ca. 0.5 g) is added, followed by centrifugation for 4 minutes at 5000 rpm.

STEP 3: ACN layer wash

The ACN phase is separated and washed twice with 12 mL of *n*-hexane. The *n*-hexane layer is discarded, and the washed acetonitrile phase is evaporated almost to dryness. 2 ml of ACN is added to the samples. The ACN phase is washed twice with 3 mL of *n*-hexane. The hexane phase is again discarded, and the ACN phase is evaporated to dryness, and the samples are dissolved with 1 mL of UHQ water.

STEP 4: Solid phase extraction and solvent exchange

The samples are extracted using SPE cartridges (e.g. Oasis HLB SPE 60 mg 3 cc is well established, but C18 could also be used). The cartridges are conditioned with 1 mL methanol followed by 1 mL UHQ water prior to sample loading. The cartridges are washed with 450 µL of 5 % methanol, and the analytes are eluted with 2 x 450 µL of 1 % formic acid in methanol. The samples are evaporated to dryness, and reconstituted in 400 µL methanol:water 1:1 (v/v). The samples are filtrated with 0.20 µm filter prior the instrument analysis.

The full sample preparation procedure is demonstrated in Figure 2.

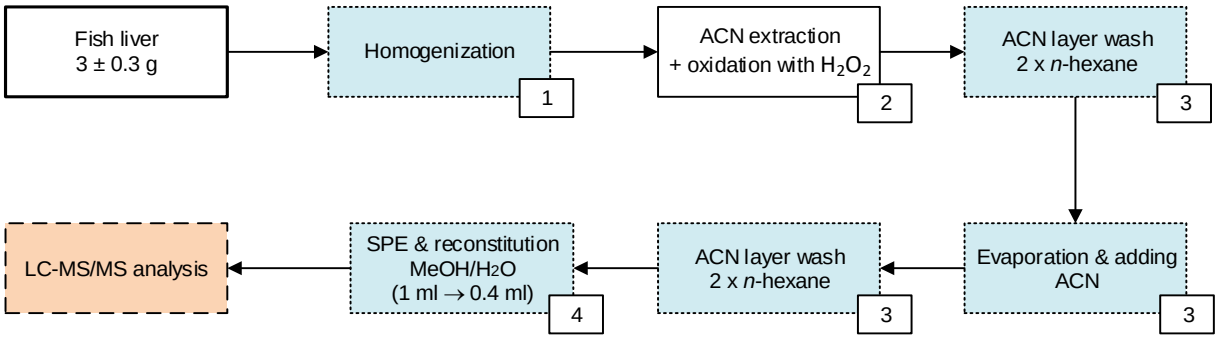


Figure 2. Sample flow chart for fish liver samples

CALIBRATION STANDARDS

All calibration standards are prepared in a blank matrix, which are a pre-treated as described above. The blank matrix should be as similar as possible to the sample matrix. The target chemicals are spiked into cod extracts after the sample preparation procedure. It is recommended to use a six-point calibration curve (e.g. range of 0.2-5 ng/g).

INSTRUMENT METHOD

Due to trace levels of analytes and the complex biological matrices discussed in this ROP, it is highly recommended to use LC-MS/MS technique to achieve selective and sensitive analysis. Atmospheric pressure ionization techniques, such as chemical ionization (APCI) and electrospray ionization (ESI) are suitable for sufficient ionization of the target chemicals. The mass spectrometer is operated in the positive ion MRM mode.

The applied LC-MS/MS method should be optimized using reference standards of the target chemicals. The reference standards should be used for optimizing the MRM transitions for the MS method. MRM transitions of protonated phenylarsenic chemicals discussed in this ROP are considered to be specific. The optimal collision energies for the target arsenic compounds should be optimized for each instrument and at least two MRM transitions should be used. The product ion with the most intense abundance should be used as the quantifier ion (Q), and the ion with lower abundance should be used the qualifier ion (q). The quantifier and qualifier transitions of protonated molecules of target chemicals are presented in Table 1.

Table 1. Example of transitions formed using ESI

Chemical	Reaction (m/z)	
	Quantifier (Q)	Qualifier (q)
Phenarsazinic acid 10-methyl-5H-phenarsazin-10-oxide	276 → 230 274 → 242	276 → 154 276 → 167
Diphenylarsinic acid	276 → 230	276 → 230
Methyldiphenylarsine oxide	261 → 154	261 → 230
Triphenylarsine oxide	323→ 227	323 → 154

The LC separation should be done using reversed-phase liquid chromatography (RPLC) with a C18 column, using a mobile phase gradient of organic and aqueous eluents. Organic acid (e.g. formic acid, acetic acid) should be used as an additive in both organic and aqueous eluents to enhance the ionization processes of target chemicals. Linear gradient elution should be applied for separation of target chemicals. For example, the following gradient can be used: 0.1 % formic acid in water (A) and 0.1 % formic acid in ACN (B) as eluents, and the gradient is run from 5 % B at 0 min to 100 % B at 5 min. After this the B eluent is kept at 100 % for 2 min and at 5 % for 2 min. The flow rate is 0.5 mL/min, and the injection volume is 5µL. Example of extracted ion chromatograms (EICs) of target chemicals spiked in fish matrix is presented in Figure 3.

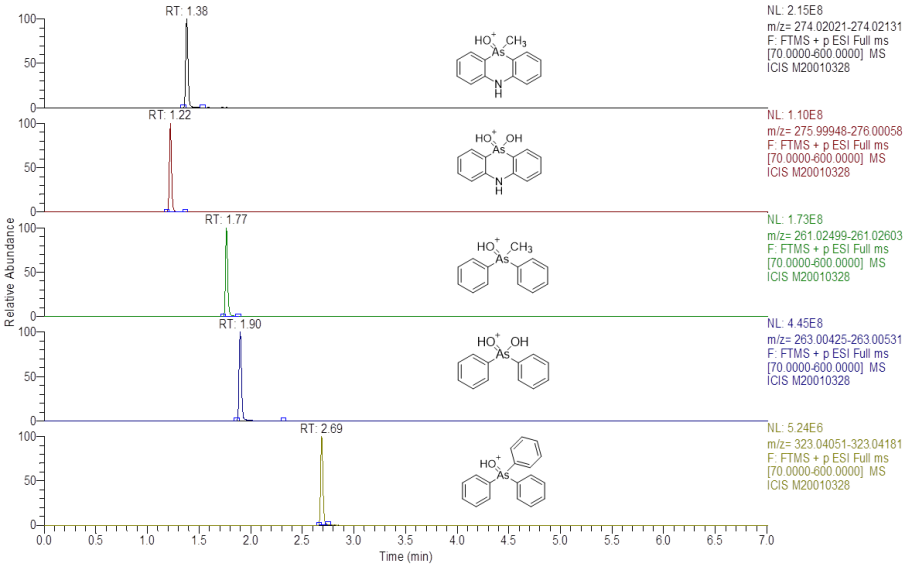


Figure 3. EICs ($[M+H]^+$) of target analytes

CRITERIA FOR IDENTIFICATION

The reliable qualitative identification of the detected chemicals is based on EU guidelines[2]. In LC analysis, the retention time of the identified compound should not differ more than ±0.2 min from the calibration standard sample. In MS/MS, the identification is based on measurement of the quantifier (Q) and the qualifier (q) ions formed from the protonated target molecule. For each target chemical detected in the samples, the ion ratio between the qualifier ion and the quantifier ion is calculated and compared to that of the reference standard. The maximum permitted tolerance for

the ion ratios of the q and Q ions must fall within a certain tolerance window. Relative intensities (% of base peak) and permitted tolerances are presented in Table 2.[2] If the q/Q-ratio does not fulfill the tolerance criteria, the identification is not considered reliable, and the data will be rejected.

Table 2. Relative intensities and permitted tolerances[2]

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

For example, if the relative intensity (% of base peak) of calibration standard is 87.4 %, the maximum permitted tolerance is 87.4 ± 17.5 % (maximum relative tolerance ± 20 %).

Limit of quantification (LOQ) values for target chemicals should be established using institute's own method validation procedures.

Conclusion

This ROP describes the method recommended for use in monitoring the marine biota living in the vicinity of CWA dumpsites for environmental risk assessment purposes. Measured concentrations of degradation products of CWA-related phenylarsenic chemicals in fish can also be combined with evaluation of biological indicators, such as increased enzyme activities and fish health status. Combining chemical and biological indicators provides comprehensive risk assessment when evaluating the impact on dumped chemical munitions on fish.

References

[1] Detection of chemical warfare agent related phenylarsenic compounds and multi-biomarker responses in cod (*Gadus morhua*) from munition dumpsites, *Mar.Environ. Res.* 162, 105160, 2020

[2] COMMISSION DECISION of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Official Journal of the European Communities, 2002/657/E, pp. 36, 2002

[3] Niemikoski *et al*. Identification of Degradation Products of Sea-Dumped Chemical Warfare Agent-Related Phenylarsenic Chemicals in Marine Sediment, *Anal. Chem.* 92, 7, 4891-4899, 2020

[4] Niemikoski *et al*. Studying the metabolism of toxic chemical warfare agent-related phenylarsenic chemicals in vitro in cod liver, *J. Haz. Mat*. 391, 5, 122221, 2020

Change history

1.0	30.3.2020	First edition
1.1	20.5.2021	Definition of the document was changed from SOP to ROP.

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