Recommended Operating Procedure (ROP)

Aim of ROP (tick box)

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

33. Chemical analysis of sea-dumped explosives in sediment

version 1.0

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Scope

This Recommended Operation Procedure (ROP) describes analysis of sea-dumped explosives in sediment and includes sample preparation, analytical methods, and the evaluation of the analytical result.

Summary of the method/ROP

This method is based on previously published methods.[1-7] The ROP utilizes optimized sample preparation for gas chromatography–electron ionization tandem mass spectrometry (GC–EI/MS/MS) technique 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 based on the most common military munitions, mainly TNT-fillled munitions.

Safety aspects

General safety aspects conducting laboratory work must be followed when handling any chemicals. Appropriate Safety Data Sheets (SDS's) should be available. Samples have to be treated as any other samples containing hazardous chemicals according to laboratory's safety instructions. Proper waste disposal procedures must be followed. Explosives can detonate by heat or mechanical impact, especially at high concentration levels (> 10 %).

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 position of the sampling site, the person(s) who did the sampling, and the name of the company 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 sample container markings so that every sample can be tracked.

Methods

Target Compounds

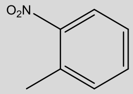
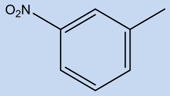
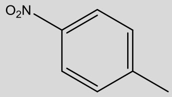
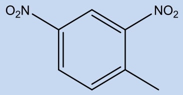
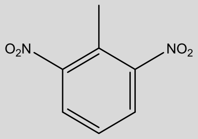
Quantitative chemical analyses of target energetic compounds are needed to prove presence/absence of dumped munitions in the marine environment.

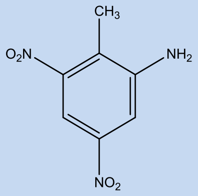
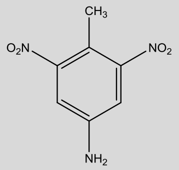
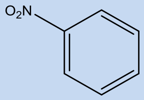
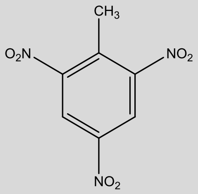
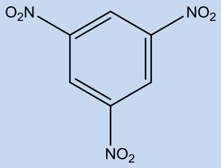
Target energetic compounds , which have been dumped in significant amounts in the Baltic Sea, and therefore were analyzed, are:

- 2-Nitrotoluene
- 3-Nitrotoluene
- 4-Nitrotoluene
- 2,4-Dinitrotoluene
- 2,6-Dinitrotoluene
- 2-Amino-4,6-dinitrotoluene
- 4-Amino-2,6-dinitrotoluene
- Nitrobenzene
- 2,4,6-Trinitrotoluene
- 1,3,5-trinitrobenzene

Table 1 summarizes the names of the analyzed compounds, their CAS numbers, acronyms, and structures.

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

#	**Chemical (acronym)** **CAS**	**Structure**	**Description**
2-NT	2-Nitrotoluene 88-72-2		*Explosive related*
3-NT	3-Nitrotoluene 99-08-1		*Explosive related*
4-NT	4-Nitrotoluene 99-99-0		*Explosive related*
2,4-DNT	2,4-Dinitrotoluene 121-14-2		*Explosive*
2,6-DNT	2,6-Dinitrotoluene 606-20-2		*Explosive*

2-ADNT	2-Amino-4,6-dinitrotoluene 35572-78-2		*Explosive related*
4-ADNT	4-Amino-2,6-dinitrotoluene 19406-51-0		*Explosive related*
NB	Nitrobenzene 98-95-3		*Explosive related*
TNT	2,4,6-Trinitrotoluene (TNT) 118-96-7		*Explosive*
1,3,5-TNB	1,3,5-trinitrobenzene 99-35-4		*Explosive*

Sample Preparation

In most cases, the sediment samples arrive frozen on dry ice at the analytical laboratory. Blank sediment samples should be collected at sea at a safe distance from the dumping area.

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 shown in **Figure 1**. This ROP describes the analysis of energetic compounds from the sediment portion.

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.

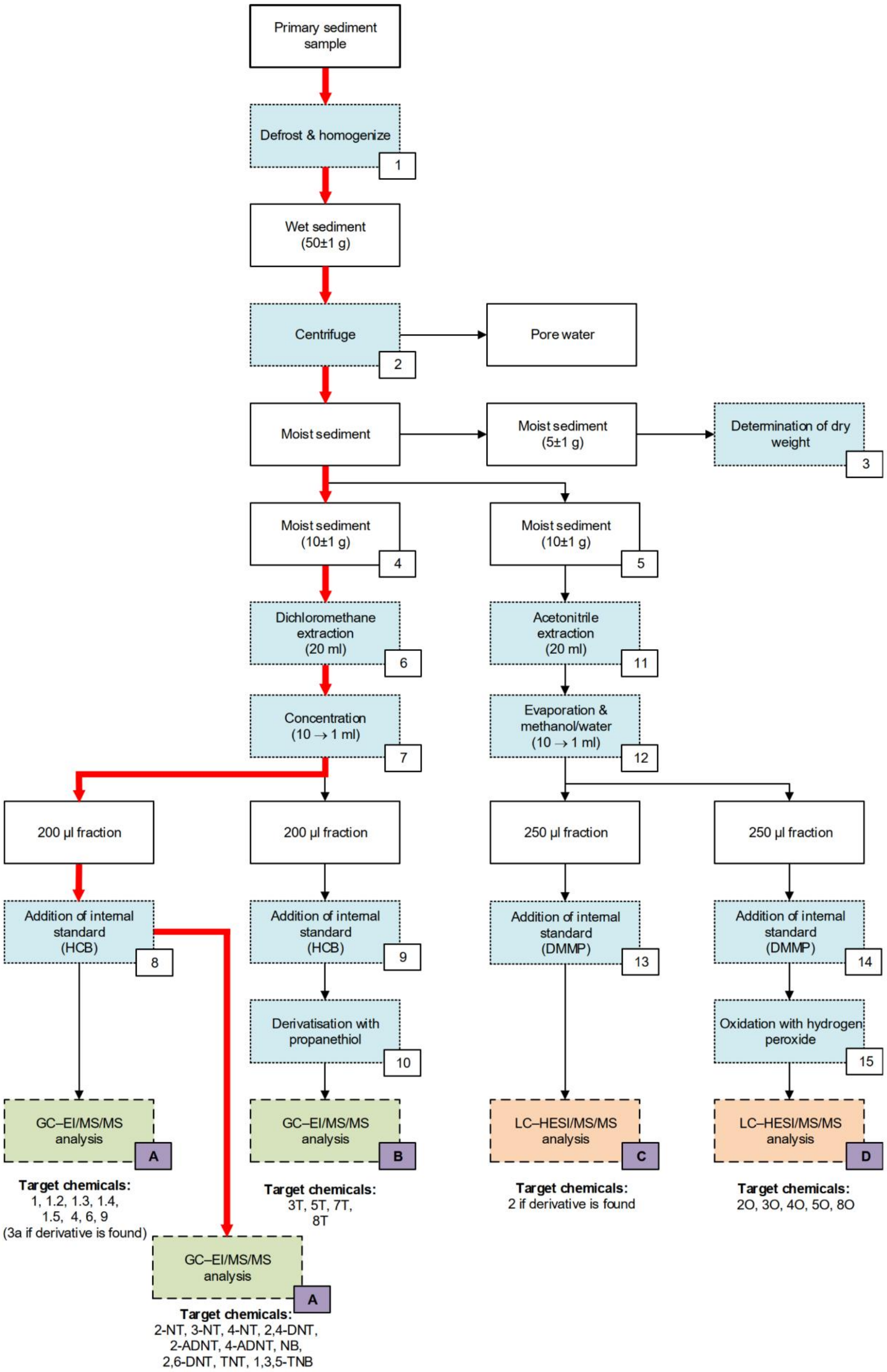


Figure 1. Sample preparation flow-chart for the sediment samples. Analyzed sample fractions are numbered **A** through **D**. The sample preparation steps 1 through 8 are described in detail in the text. The sample preparation and analysis procedures for CWA analytes, including derivatization reactions (steps 9 through 15), is described in ROP "Chemical analysis of sea-dumped chemical warfare agents in sediment, and pore water".[8]

STEPS 1 and 2: Removal of Pore Water

The sediment is homogenized by mixing thoroughly with a plastic spoon before taking an aliquot. A 50±1 g-portion of the sediment is weighed into a Falcon tube. The remaining sediment should be stored in a freezer for possible later use. Next, the sample is centrifuged for 3 minutes at 1000 x*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 approx. 3 - 5 spoonfuls (10 – 15 ml in total) in order to represent the whole sediment sample.

STEP 6: Dichloromethane (DCM) 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 (RT, 1000 rpm). After this, the sample is centrifuged for 3 minutes at 1000 x *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 (intact CWAs and explosive) and 9 (derivatized CWAs if analyzing those)) and 10 µl of internal standard (hexachlorobenzene, HCB, c = 1 µg/ml) is added to both.

Note: HCB is not the optimal ISTD for the explosives (1,3 DNT would be better), but HCB is used mainly to correct for instrument errors (injection).

This fraction, **A (vial 8),** will be analyzed by GC–MS/MS for **intact chemicals (2-NT, 3-NT, 4-NT 2,4-DNT, 2,6-DNT, 2-ADNT, 4-ADNT, NB, TNT, and 1,3,5-TNB**).

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**):

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

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.

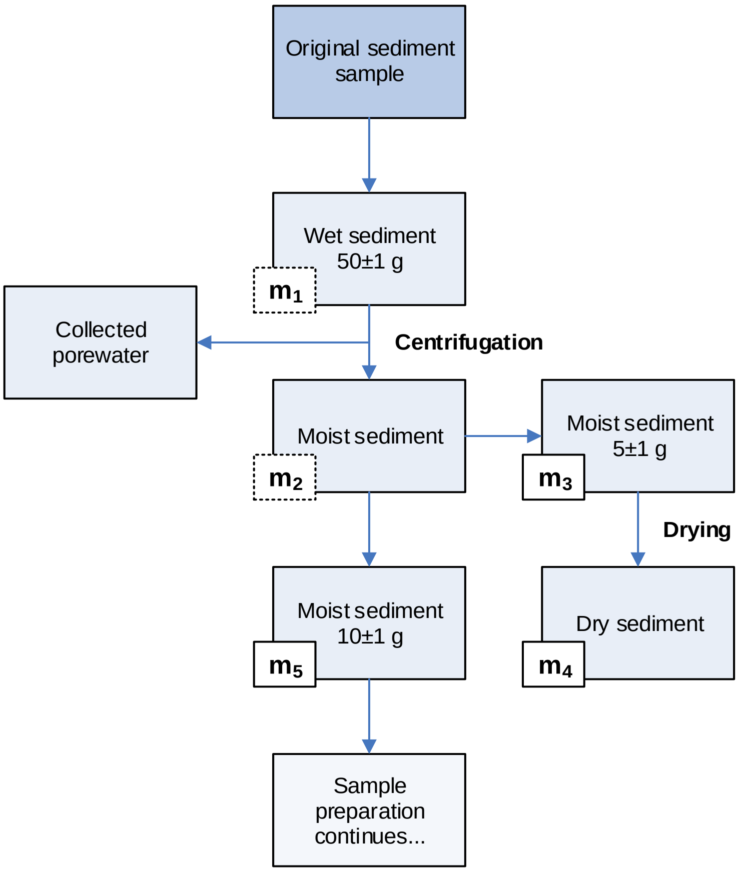


Figure 2. Flowchart describing the weighing process.

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:

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

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). All standards are made by spiking the chemicals into blank sediment extracts (step 8), which have been identically prepared.

For the GC-MS/MS technique, a six to eight-point calibration curve (ca. 0.5–100 pg/µl, depending on expected sample concentrations) is applied.

Mass spectrometric methods

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

GC–MS/MS fraction is analyzed using the following analysis method or similar. This standard operation procedure describes an 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 program is the following:
Starting temperature 45 °C (3.3 min hold) → 7 °C/min to 210 °C → 20 °C/min to 250 °C (1 min hold) → 10 °C/min to 280 °C (2.1 min hold). Total run time is 35 minutes.

Multimode inlet at PTV mode/splitless (splitless time 1min) is used with following temperature program: Starting temperature 60 °C →200 °C/min to 250 °C. Injection volume is 1 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 transferred to any corresponding GC-MS/MS analytical system.

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

#	Chemical (acronym)	Reactions (*m/z*) and collision energies (eV) in brackets		
		Quantifier Q	Qualifiers q	
NB	Nitrobenzene	77.0 → 51.0 (18)	123.0 → 51.0 (42)	123.0 → 77.0 (15)
2-NT	2-Nitrotoluene	120.0 → 92.0 (8)	120.0 → 65.0 (15)	65.0 → 39.0 (20)
3-NT	3-Nitrotoluene	91.0 → 65.0 (13)	137.0 → 65.0 (32)	137.0 → 91.0 (13)
4-NT	4-Nitrotoluene	91.0 → 65.0 (12)	137.0 → 107.0 (5)	137.0 → 91.0 (17)
2,6-DNT	2,6-Dinitrotoluene	165.0 → 63.0 (28)	165.0 → 90.0 (15)	89.0 → 63.0 (22)
2,4-DNT	2,4-Dinitrotoluene	89.0 → 63.0 (20)	165.0 → 119.0 (5)	165.0 → 63.0 (39)
1,3,5-TNB	1,3,5-Trinitrobenzene	75.0 →74.0 (25)	75.0 → 38.0 (47)	120.0 → 74.0 (18)

TNT	Trinitrotoluene	89.0 →63.0 (18)	210.0 → 164.0 (5)	210.0 → 193.0 (10)
4-ADNT	4-amino-2,6-dinitrotoluene	180.0 →78.0 (25)	104.0 → 77.0 (10)	197.0 → 180.0 (5)
2-ADNT	2-amino-4,6-dinitrotoluene	197.0 →180.0 (5)	104.0 → 77.0 (12)	78.0 → 52.0 (12)
HCB (ISTD)	Hexachlorobenzene	284.0 → 214.0 (35)	284.0 → 142.0 (50)	284.0 → 249.0 (25)

Criteria for identification

In the described methods, 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."

The use of the identification criteria is illustrated in Figure 3 for GC–EI/MS/MS data using two different level calibration standards. The retention time difference between simulated reference sample (reference) and the simulated real sample (sample) is within the allowed limit ±0.1 min. The ratios of the two qualifier reactions (165→119 and 165→63) compared to the quantifier reaction (89→63) were 76.3 % and 81.6 %, respectively. The allowed limits for these ratios were 73.4±14.7 % and 83.1±16.6 %, respectively. Based on these two criteria the example identification in Figure 4 can be accepted.

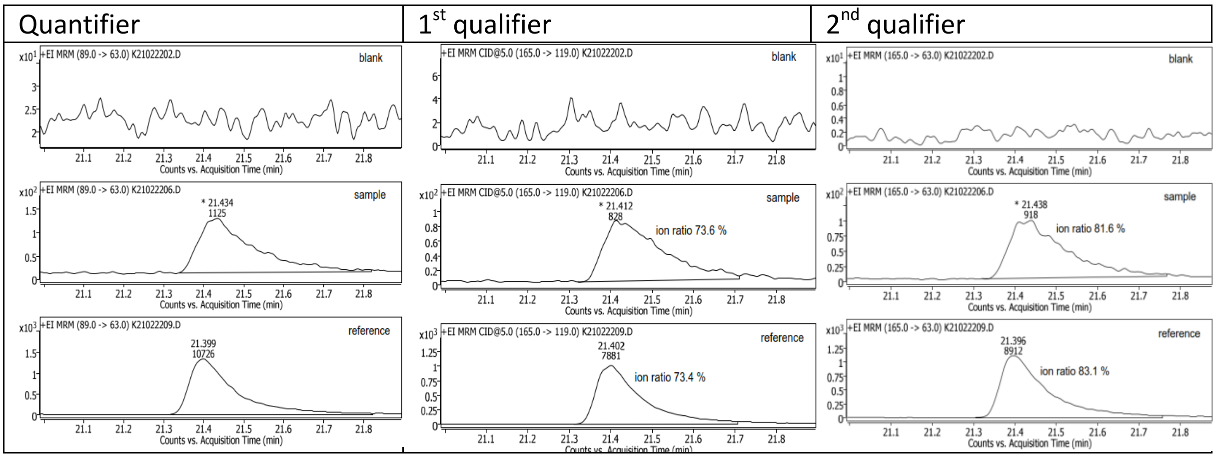


Figure 3. Demonstration of identification criteria for 2,4-dinitrotoluene (2,4-DNT) using GC–EI/MS/MS technique. Presented MRM chromatograms are for blank sediment sample (blank), simulated contaminated sediment sample (sample), and a 50 ng/ml reference sample (reference).

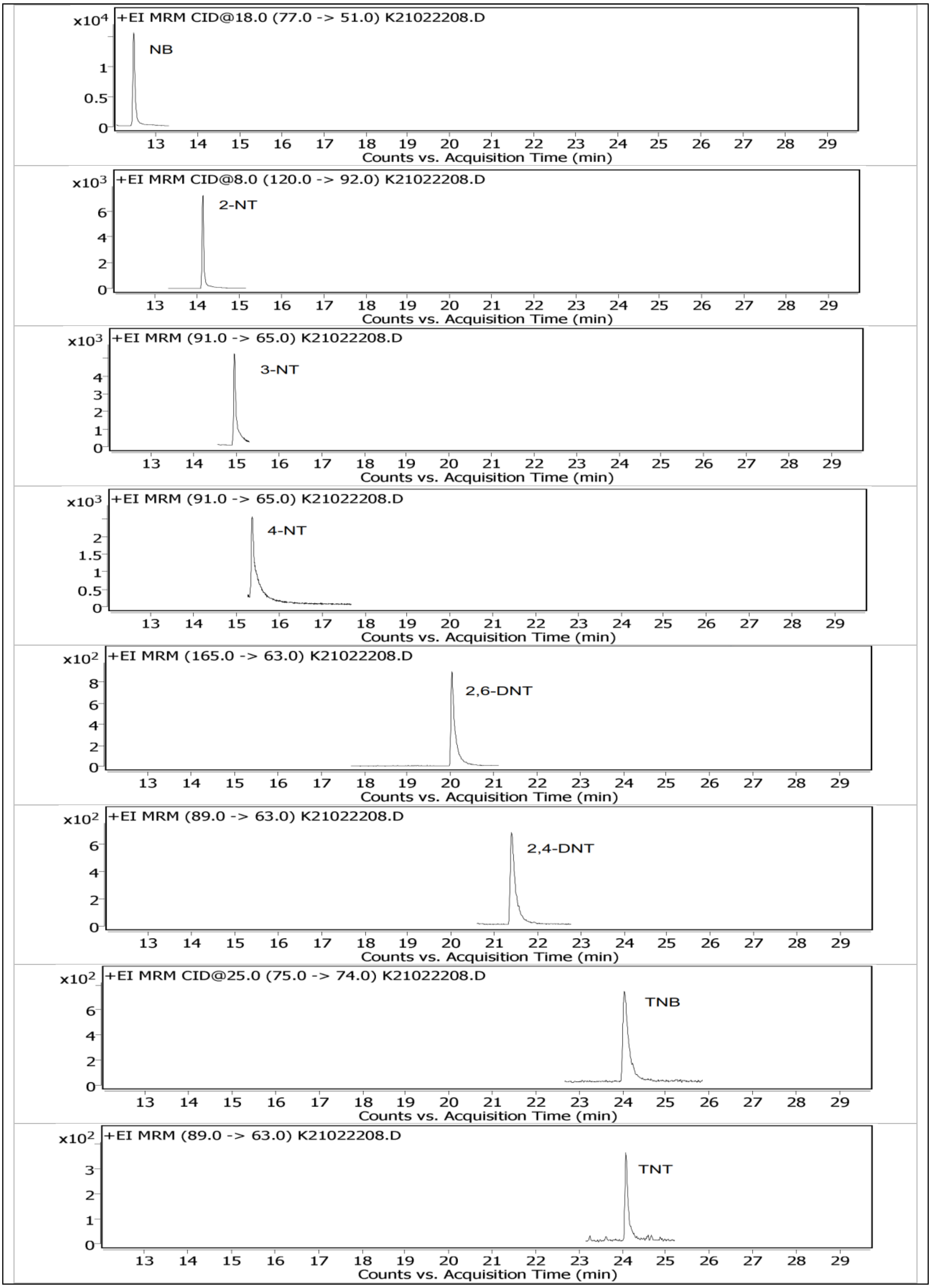
Quality control

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 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(s) used for the analysis should be standard solution(s) containing all monitored chemicals at the concentration levels of ca. 10-25 pg/µl, depending on the expected concentrations of the analytes in the samples. Control samples are analyzed once or twice during the sample batch, depending on the total number of samples. Examples of relevant runs are presented in Figure 9.

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) and quality blank (similar sample preparation without sediment).


x10⁴
+EI MRM CID@18.0 (77.0 -> 51.0) K21022208.D
NB
1
0.5
0
13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
Counts vs. Acquisition Time (min)
x10³
+EI MRM CID@8.0 (120.0 -> 92.0) K21022208.D
2-NT
6
4
2
0
13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
Counts vs. Acquisition Time (min)
x10³
+EI MRM (91.0 -> 65.0) K21022208.D
3-NT
4
3
2
1
0
13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
Counts vs. Acquisition Time (min)
x10³
+EI MRM (91.0 -> 65.0) K21022208.D
4-NT
2
1.5
1
0.5
0
13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
Counts vs. Acquisition Time (min)
x10²
+EI MRM (165.0 -> 63.0) K21022208.D
2,6-DNT
8
6
4
2
0
13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
Counts vs. Acquisition Time (min)
x10²
+EI MRM (89.0 -> 63.0) K21022208.D
2,4-DNT
6
4
2
0
13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
Counts vs. Acquisition Time (min)
x10²
+EI MRM CID@25.0 (75.0 -> 74.0) K21022208.D
TNB
6
4
2
0
13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
Counts vs. Acquisition Time (min)
x10²
+EI MRM (89.0 -> 63.0) K21022208.D
TNT
3
2
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13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
Counts vs. Acquisition Time (min)

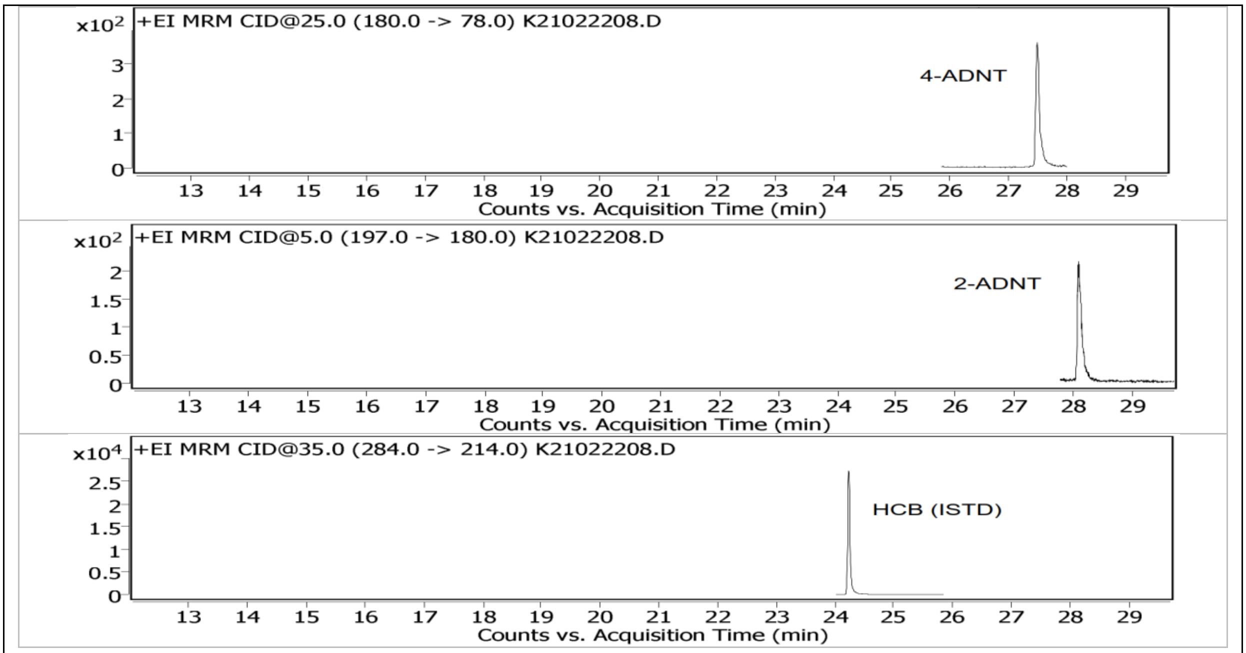

Figure 9. GC–EI/MS/MS MRM chromatograms for ca. 25 pg/µl standards.

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 methods. The lower limit of quantitation (LLOQ) values for GC-MS/MS range from 1 to 5 ng/ml.

Conclusions (if applicable)

This ROP describes sample preparation and analytical methods for analysis of explosives related chemicals in sediment utilizing GC-MS/MS technique. The LLOQ values for GC-based methods are generally from 1 to 5 ng/ml.

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] SW-846 Test Method 8095: Explosives by Gas Chromatography

[8] Hakulinen *et al*., Chemical analysis of sea-dumped chemical warfare agents in sediment, and pore water, DAIMON2 Project

[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	20.05.2021	Definition of the document was changed from SOP to ROP.

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