Standard Operating Procedure (SOP)

Aim of SOP (tick box)

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

23. Extraction and analysis of explosives and metabolites in fish bile via HPLC-QQQ-MS

version 1.1

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Scope

This Standard Operating Procedure describes the extraction and identification of the nitroaromatic explosive compounds 2,4,6-trinitrotoluene (TNT), RDX and HMX as well as the TNT degradation products 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2,4-dinitrotoluene (2,4-DNT) and 2,5-dinitrotoluene (2,5-DNT) in fish bile via liquid chromatography-tandem mass spectrometry (LC-MS/MS).

The gallbladder contains bile, a green to yellow fluid produced in the liver which is involved in the digestive process. Investigating the contamination of bile samples with explosive compounds provides a picture of the uptake of contaminants most likely processed by the fish liver. Since the liver is known as a detoxification organ and contains enzymes involved in the biodegradation of contaminants, metabolites are also found in bile[1]. It is important to consider that components detected in the bile are temporarily found there before being excreted from the gallbladder during the digestive process.

Summary of the method/SOP

The bile sampled onboard is immediately frozen and nitroaromatic explosive compounds and degradation products are later extracted with acetonitrile (ACN). The analysis is performed by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and ionization was conducted in negative atmospheric pressure chemical ionization (APCI) mode. The concentrations of identified compounds are calculated by calibrations using an internal standard.

Safety aspects

Depending on the chemicals to be analyzed, the corresponding risk assessments have to be considered before starting work. If further instructions are required, contact the local safety officer or the laboratory manager.

Documentation

An orderly pipetting scheme should be followed to avoid pipetting errors. If the required amount of bile for extraction is not available, this must be noted in the documentation. Similarly, loss of sample material, e.g. by overturning reaction tubes, should be documented. The results should be transferred into a suitable format (e.g. Excel spreadsheet) for quantification. Using an internal standard, a recovery rate can be calculated and applied to the target components.

Methods

Equipment:

- Pipettes, volume adjustable including tips.
- Reaction tubes 1.5 mL volume, e.g. Eppendorf
- Analytical brown glass vials of 1.5 mL, with and without glass inserts
- Analytical syringe, 100 µL
- Refrigerated centrifuge for 1.5 mL reaction tubes
- N_2 reduction unit
- Acclaim E2 explosive column, 2.2 µm particle size, 2.1 mm diameter, 150 mm length (Thermo Fisher)
- Nitrogen supply

Chemicals:

Chemical	**CAS-Number**	**Supplier**
Acetonitrile (99.9%)	75-05-8	Merck
Ammonium acetate (NH_4Ac)	631-61-8	Sigma-Aldrich
Acetic acid (99%)	64-19-7	Merck
Methanol (MeOH, 99.9%)	67-56-1	Merck
MilliQ water (15 MΩ cm)		
Calibration standards	**CAS-Number**	**Supplier**
1,4-dinitrobenzene (1,4-DNB, internal standard))	100-25-4	Sigma-Aldrich
Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)	2691-41-0	AccuStandard
Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)	121-82-4	AccuStandard
2,4,6-trinitrotoluene	118-96-7	AccuStandard
2-amino-4,6-dinitrotoluene	35572-78-2	AccuStandard
4-amino-2,6-dinitrotoluene	19406-51-0	AccuStandard
2,4-dinitrotoluene	121-14-2	NEOCHHEMA
2,5-dinitrotoluene	619-15-8	NEOCHHEMA

- Eluent A: H_2O (10 mM NH_4Ac + 2.7 mL acetic acid, pH 4)
- Eluent B: MeOH (10 mM NH_4Ac + 2.7 mL acetic acid)

Sample material:

Bile is extracted from the gallbladder of desired fish species. So far, bile from flatfish species such as dab (*Limanda limanda*), European flounder (*Platichthys flesus*) and plaice (*Pleuronectes platessa*) was investigated. In principle, any bile sample taken from other fish species can be used for the extraction process and should be frozen immediately after sampling at -20 °C.

Bile extraction procedure:

An aliquot of 25 µL of the original bile sample is transferred into a 1.5 mL Eppendorf vial using a 100 µL Eppendorf pipette with disposable tips. For every 20 samples one vial is filled with 25 µL of Milli-Q water instead of bile as an extraction blank and treated like a sample for the following steps.
The sub-samples are supplemented with 5 µL of internal standard 1,4-dinitrobenzene (1,4-DNB; 10 ng/µL). Thereafter, 1 mL of cold (~4°C) ACN is added to each vial as extraction solvent and the solution is mixed vigorously for 30 seconds on a Vortex mixer. The samples are left to incubate for 10 minutes at 4 °C in the dark and are subsequently centrifuged for 10 min at 6000 rpm at 4 °C. The supernatant from each sample is right away transferred into 1.5 mL brown glass vials and reduced to circa 200 µL under a stream of nitrogen.
The reduced sample is transferred into a new 1.5 mL brown glass injection vial with a glass insert and again reduced to a total volume of approximately 50 µL. The final volume should be measured with an analytical syringe. From this concentrated sample 5 µL are directly afterwards injected on the HPLC-MS for analysis of explosives compounds. If samples have to be stored for short periods of time before analysis, these are kept in the dark at -20 °C until further treatment.

HPLC-MS procedure:

Bile extracts are dissolved in Acetonitrile and 5 µL of the respective solution is injected into the HPLC device for separation and subsequent analysis with QQQ-MS.

The HPLC gradient separation is conducted as follows: 5 minutes isocratic at 45 % B, increase to 60 % B at 5 minutes, followed by a gradient from 60 to 95 % B from 5 to 40 minutes with a subsequent reconditioning of the column at 45 % B for 5 minutes. The flow rate is kept constant at 0.3 ml/min and column is kept at 22 °C.

Ionization is done in negative atmospheric pressure chemical ionization (APCI) mode. The detection of target analytes is conducted in Multiple Reaction Monitoring (MRM) mode with concurrent recording of a full scan Enhanced Mass Spectrum (EMS). Ionization energies are optimized using commercially available standard substances. Conditional screening for nitroaromatic compounds is performed via Neutral Loss (NL) scanning for loss of individual nitro groups (m/z 46) and consecutive acquisition of Enhanced Product Ion (EPI) spectra of the nitro group containing compound. Analysis of explosive compounds from marine biota samples is described in Koske et al.[1].

Prerequisites for a good separation in HPLC are an efficient and clean extraction, an intact column and the correct adjustment of injection volume, solvent gradient, column, pressure and temperature curve (HPLC method). Interference peaks in chromatography can arise either from the sample matrix due to incomplete purification of the extracts, or from contamination of the chemicals and equipment used.

Quantification:

The amount of the analyte present in the sample is quantified using the internal standard 1,4-DNB, which is added to each sample before extraction (5µL of 10 ng/µL 1,4-DNB). Response factors for individual substances are determined through external standard calibration. The standard mix used for calibration contains the following substances: 1,4-DNB (internal standard); HMX; RDX; TNT; 2,4-DNT; 2,5-DNT; 2-ADNT; 4-ADNT and is analysed in concentrations of: 1; 0.5; 0.1; 0.05; 0.01; 0.005; 0.001 ng/µL and is routinely measured in duplicate. The slope of the standard curve was obtained by linear regression. The detection limits (LOD) and quantification limits (LOQ) are shown in Table 1, retention times of the analytes are given in Table 2.

Table 1: Limit of detection (LOD) and limit of quantification (LOQ) obtained for different explosive compounds.

Compound	LOD [ng/mL bile fluid]	LOQ [ng/ml bile fluid]
HMX	0.4	2.4
RDX	1.2	4.4
TNT	2	7.6
2,4-DNT	13.2	46.4
2,5-DNT	9.2	33.2
2-ADNT	3.2	12.4
4-ADNT	2.8	10.8

Table 2: Retention times (minutes) and elution order of analytes using the equipment and parameters described in this SOP.

Compound	Retention time [min]
HMX	6.32
RDX	9.28
1,4-DNB	9.88
TNT	12.12
2,5-DNT	13.03
2,4-DNT	13.43
4-ADNT	15.10
2-ADNT	15.69

References

(1) Koske, D.; Straumer, K.; Goldenstein, N. I.; Hanel, R.; Lang, T.; Kammann, U. First Evidence of Explosives and Their Degradation Products in Dab (Limanda Limanda L.) from a Munition Dumpsite in the Baltic Sea. *Mar. Pollut. Bull.* **2020**, *155*, 111131. https://doi.org/10.1016/j.marpolbul.2020.111131.

Change history

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