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Method Improvement for the Determination of TNT and its Degradation Products in Marine Sediment by GC–MS/MS

Advanced Spectroscopy in Chemistry

Master's thesis

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Abstract:
A significant amount of chemical and conventional weapons were dumped into seas and oceans over the last century, especially after World War II. These dumped munitions expose entire marine ecosystems to danger, making long-term environmental monitoring essential to understand and address their impacts. The literature reviewed in this work showed that numerous international projects have investigated the distribution, ecological effects, and mitigation strategies related to sea-dumped munitions. The assessment of published research studies on sediment sample analysis revealed that solid-liquid extraction (SLE) and solid-phase extraction (SPE) are among the most commonly employed methods as well as gas and liquid chromatography separation techniques coupled with mass spectrometry. Although the available studies contribute significantly to the monitoring of munition contamination in marine environments, there is still a gap when it comes to the determination of TNT and its main degradation compounds in marine sediment by gas chromatography-tandem mass spectrometry (GC–MS/MS). Therefore, to fill this research void, the experimental part of this study aimed to improve the reference method from the Blue Book (collection of the Recommended Operating Procedures for Analysis in the Verification of Chemical Disarmament), with particular focus on sample preparation optimization and the refinement of the Multiple Reaction Monitoring (MRM) conditions. SLE with 50:50 ethyl acetate-dichloromethane and optimized parameters, yielded the highest overall recoveries of the target analytes (average recovery of 51.9%). The SPE experiments showed that the analytes were not recovered from the tested cartridges, meaning that further investigations are needed to obtain a sample preparation clean-up.

List of Abbreviations

1,3-DNB	1,3-dinitrobenzene
2,4-DNA	2,4-dinitroaniline
2,4-DNT	2,4-dinitrotoluene
2,6-DNT	2,6-dinitrotoluene
2-ADNT	2-amino-4,6-dinitrotoluene
2-NT	2-nitrotoluene
3,4-DNT	3,4-dinitrotoluene
3,5-DNA	3,5-dinitroaniline
3,5-DNT	3,5-dinitrotoluene
3-NT	3-nitrotoluene
4-ADNT	4-amino-2,6-dinitrotoluene
4-NT	4-nitrotoluene
4-NT d7	deuterated 4-nitrotoluene
ACN	acetonitrile
ASE	accelerated solid extraction
CE	collision energy
$CHCl_3$	chloroform
CHEMSEA	Chemical Munitions, Search and Assessment
CW	chemical weapon
CW/TPR	Carbowax/templated resin
CWA	chemical warfare agent
DAIMON	Decision Aid for Marine Munitions
DCM	dichloromethane
DMDNB	2,3-dimethyl-2,3-dinitrobutane
ECD	electron capture detection
EGDN	ethylene glycol dinitrate
EI	electron ionization
ESI	electrospray ionization
ETN	erythritol tetranitrate
EtOAc	ethyl acetate
EtOAc-DCM	ethyl acetate-dichloromethane
GC	gas chromatography
HCB	hexachlorobenzene
HELCOM	Baltic Marine Environment Protection Commission
HMTD	hexamethylene triperoxide diamine
HMX	cyclotetramethylene-tetranitramine

HPLC	high performance liquid chromatography
HRMS	high-resolution mass spectrometry
IO PAN	Institute of Oceanology Polish Academy of Sciences
ISTD	internal standard
KH	Kolberger Heide
Kow	octanol/water partition coefficient
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MALDI	matrix-assisted laser desorption/ionization
MC	munition compound
MeOH	methanol
MERCW	Modelling of Ecological Risks Related to Sea-Dumped Chemical Weapons
MgO_4	magnesium sulfate
MMinE-SwEEPER	Marine Munitions in Europe – Solutions with Economic and Ecological Profits for Efficient Remediation
MODUM	Towards the Monitoring of Dumped Munition Threat
MRM	Multiple Reaction Monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MUNIMAP	Baltic Sea Munitions Remediation Roadmap
MUNIRISK	Mitigation of Risks Due to Submerged Munitions for a Sustainable Development of the Baltic Sea
Na_2SO_4	sodium sulfate
NATO	North Atlantic Treaty Organization
NB	nitrobenzene
NG	nitroglycerin
PA	polyacrylate
PDMS-DVB	polydimethylsiloxane/divinylbenzene
PETN	pentaerythritol tetranitrate
ppb	parts per billion
ppm	parts per million
PS-DVB	polystyrene-divinylbenzene
PTV	Programmable Temperature Vaporization
QC	Quality Control
RDX	1,3,5-trinitro-1,3,5-triazine
RR	relative recovery
R-salt	hexahydro-1,3,5-trinitroso-1,3,5-triazine

S/N	signal-to-noise ratio
SADSPE	solvent-assisted dispersive solid-phase extraction
SE	Soxhlet extraction
SLE	solid-liquid extraction
SPE	solid-phase extraction
SPME	solid-phase microextraction
SPS	Science for Peace and Security
TATP	triacetone triperoxide
Tetryl	2,4,6-trinitrophenylmethylnitramine
TNB	1,3,5-trinitrobenzene
TNT	2,4,6-trinitrotoluene
TOF	time-of-flight
UV	ultraviolet
VERIFIN	Finnish Institute for Verification of the Chemical Weapons Convention

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1 Introduction

After the World Wars, from the late 40's to early 60's, large amounts of chemical and conventional weapons were dumped into the Baltic Sea, particularly in specific regions known as dumping sites.[1–5] Back then, sea dumping was considered a cheap and easy disposal method and it was believed that water would eventually neutralize the dangerous substances.[6] Apart from deliberately dumped at designated sites, munitions were also thrown overboard while *en route* to and from these areas. At times, entire ships sunk with their cargo, further complicating efforts to accurately map these contaminated regions.[6] These operations only stopped after the Convention on the Prevention of Marine Pollution by Dumping of Wastes and Other Matter (London Convention) came into force in 1975, but the negative impacts on the environment were yet to be fully comprehended.[2,7,8]

At least 200,000 tons of conventional munition and approximately 50,000 tons of chemical weapons (CWs) containing roughly 15,000 tons of chemical warfare agents (CWAs) were dumped in the Baltic Sea over the past decades.[1,2,6,7,9,10] CWAs are toxic chemicals used intentionally to cause harm or death (e.g. sulfur mustard) and conventional munitions (e.g. mines) are materials designed to cause damage and destruction through kinetic, explosive or incendiary force. Figure 1 shows a diver close to a mine on the seabed in the Baltic Sea.

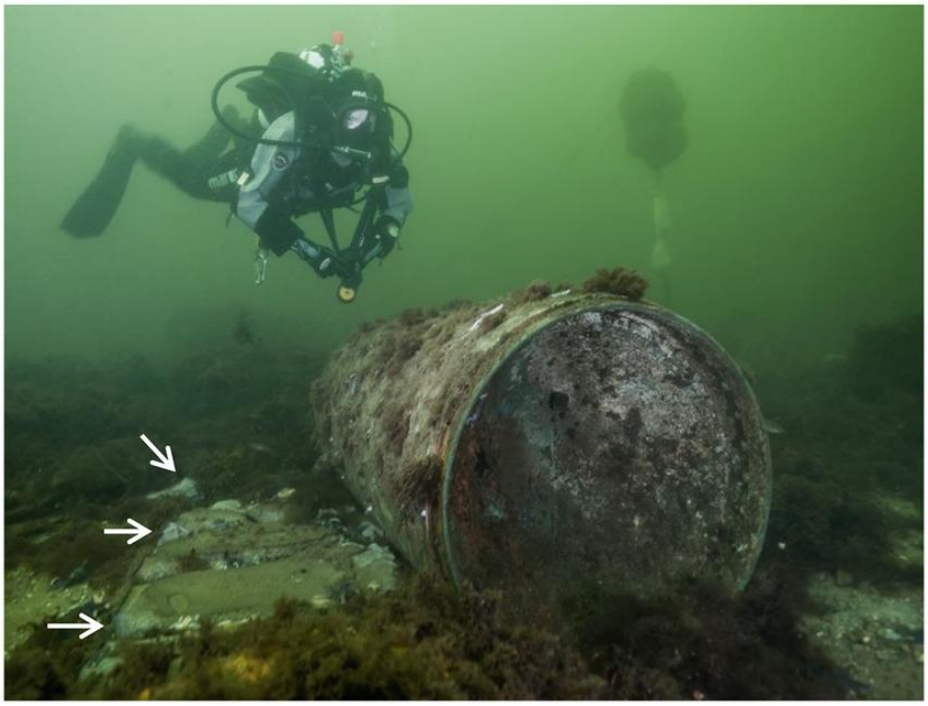


Figure 1. Diver near a German mine at the bottom of the Baltic Sea. Arrows indicate possible fragments of explosives from incomplete or partial detonation of other mines in the area.[8]

Explosives, a type of conventional munition, are materials designed to cause extensive destruction by rapidly releasing large amounts of energy.[11] 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitro-1,3,5-triazine (RDX) and pentaerythritol tetranitrate (PETN) were the most common ones used in World War II.[12] TNT was first synthesized in the 1860s but its military use only began in 1902, a few years before the outbreak of the first World War (Figure 2).[8,13]

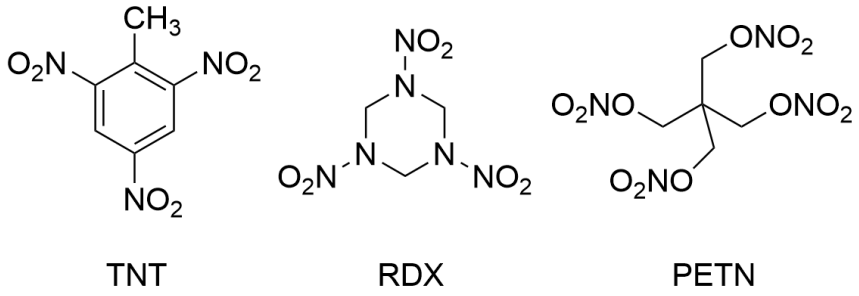


Figure 2. Structure of common explosives used in World War II.

Although these chemicals are no longer improperly discarded into seawater, the munitions dumped over the past decades still threaten the marine environment. These

toxic compounds and their breakdown products are constantly leaking into the surrounding ambience, deeply affecting marine life and the ecosystems functioning. At the present juncture, multiple factors, such as climate change and anthropogenic activities, can worsen the environmental hazards linked to sea-dumped CWs, reinforcing the need for continued surveillance in these regions.[14,15]

As well as providing up-to-date information on dumping areas, environmental monitoring is the key in developing and implementing decision support systems, policy recommendations, and analytical methods for sea-dumped munitions. Numerous projects intended to safeguard the marine ecosystems have been supported and financed over the last few decades by intergovernmental organizations and research institutes, but more efforts and resources are still required to effectively tackle this current and relevant issue.[1]

As scientific understanding of the environmental impacts caused by sea-dumped munitions has evolved, it has become evident that their adverse effects on marine biota result not only from the parent compounds but also from their degradation products. In some cases – such as TNT and its breakdown products – these degradation products can be even more toxic.[8]

These toxic compounds can be examined in various matrices, including seawater, sediment, excavated explosives, and marine organisms. Among these, sediment analysis is particularly suitable for studying TNT and its degradation products in the Baltic Sea, as these compounds exhibit low solubility in water.[16,17] As for sediment extraction, solid-liquid extraction (SLE), method in which analytes partition from a solid phase (sample) to a liquid one (organic solvent), is widely used due to its simplicity and low cost.[18]

An extraction method commonly employed for water samples and that can be further used to remove matrix interferences from sediment extracts is solid-phase extraction (SPE), clean-up method based on chromatographic separation. Chromatographic techniques such as liquid chromatography (LC) and gas chromatography (GC), coupled with varying detectors (e.g. mass spectrometry, ultraviolet/visible, electron capture) are the most applied methods for analyzing explosives in diverse sample matrices.[9,12,16,19,20] TNT and its degradation products can be successfully analyzed by both chromatographic

techniques and with regards to detection, MS detectors have greater sensitivity, molecular specificity and are less prone to interferences, making them an excellent choice for analyzing complex matrix samples, such as marine sediment.

This study was divided into two parts: literature review and experimental research. The first part aimed to survey existing literature on sea-dumped munitions, TNT microbial degradation and impacts in marine ecosystems, and analysis of organic explosives in environmental samples with special focus on sample preparation protocols and analytical methods. The second part was dedicated to improving the sample preparation procedure and optimizing the MRM method for the gas chromatography-tandem mass spectrometry (GC–MS/MS) analysis described in the "Analysis of explosives in sediment and soil" chapter of the Blue Book by the Finnish Institute for Verification of the Chemical Weapons Convention (VERIFIN).

2 Literature Review

2.1. Efforts addressing sea-dumped munitions: past and present

The dumping of CWs into seas and oceans, particularly after the World Wars, has resulted in thousands of tons of toxic material resting in the marine environment. These aging munitions are gradually degrading, releasing hazardous substances into the environment. The resulting compounds are potentially persistent, capable of bioaccumulation and may undergo further transformation into other products whose toxicity is unknown. Multiple factors impair the studies to address the risks associated with sea-dumped munition and to develop efficient remediation strategies. These include the lack of official records of dumping operations and the uncertainty regarding the exact location of the dumping sites.[21]

When these areas are of known location, recovery operations are often challenging since mechanical stress can cause sudden explosions or release even more toxic chemicals into the environment.[4] Thus, over the last two decades, several projects have been conducted to address and investigate the distribution, ecological effects, risk assessment and mitigation strategies related to sea-dumped munitions. [15,21–23]

The Baltic Marine Environment Protection Commission – also known as the "Helsinki Commission" or "HELCOM" – is an intergovernmental organization established in 1974 to protect the marine environment of the Baltic Sea from all sources of pollution. HELCOM's tasks and projects are not strictly related to sea-dumped munition but are rather wider in scope when it comes to protecting the Baltic Sea.[24]

'Modelling of Ecological Risks Related to Sea-Dumped Chemical Weapons' (MERCW) was the pioneering project initiated in 2004. Along with nine partners from Belgium, Denmark, Finland, Germany, Latvia and Russia, the consortium confirmed the existence of submerged munitions in the Bornholm Deep area and constituted the first large-scale risk assessment of sea-dumped CWA to the marine ecosystem.[21,25,26]

Following MERCW, the 'Chemical Munitions, Search and Assessment' (CHEMSEA) was a flagship project of the Baltic Sea Region Strategy from 2011 to 2014 that emerged from the need to describe chemical weapon dump sites that had been omitted or only partially included in previous investigations.[6,9] Apart from biota-related studies, 179 sediment samples were collected from five different sites and nearly one third of them contained at least one target chemical.[6] These results confirmed the presence of CWAs at the unofficial sites of Gdańsk Deep and Slupsk Furrow, supporting the mapping of munition dumping zones in the Baltic Sea.

After the issue was highlighted by the CHEMSEA findings, NATO financed the follow-up project called 'Towards the Monitoring of Dumped Munition Threat' (MODUM) within the Science for Peace and Security (SPS) Programme. This project aimed to monitor dumping sites and develop/improve identification methods for explosives and CWAs to better understand the impact of sea-dumped chemical munition on the Baltic environment.[1,7]

Then the EU funded 'Decision Aid for Marine Munitions' (DAIMON), under the Interreg Baltic Sea Region Programme 2014–2020, continued the studies of dumped munition focusing on creating decision-support tools for maritime administrations of Baltic countries for the sustainable management of munition dump sites.[7,10] Analysis of samples collected during that project are discussed later in the thesis. An extension project called

DAIMON 2 (2019-2021) was created to popularize these decision-aid tools and train pertinent authorities around the Baltic Sea.[10]

The latest ongoing projects initiated in 2024 are the 'Mitigation of Risks Due to Submerged Munitions for a Sustainable Development of the Baltic Sea' (MUNIRISK), 'Baltic Sea Munition Remediation Roadmap' (MUNIMAP), and 'Marine Munitions in Europe – Solutions with Economic and Ecological Profits for Efficient Remediation' (MMinE-SwEEPER).[27–29]

The main objective of MUNIRISK is to decisively address the risks posed by submerged munitions to protect the Baltic Sea environment and support safe maritime activities/resource development.[27] MUNIMAP's primary goal is to develop a flexible, modular roadmap for the remediation of munitions in the Baltic Sea, while the MMinE-SwEEPER project aims to advance the knowledge and expertise for marine munitions clearance in Europe.[28,29] The experimental research performed as part of this thesis will contribute towards the objectives of the MUNIMAP project.

According to HELCOM reporting system, from 1994 until 2012, over a hundred cases of chemical munitions being caught by fishermen have been reported, with most of them involving lumps of sulfur mustard.[30] When netted warfare materials were unsafe to be brought and handled on land, they were relocated to so-called emergency relocation areas, i.e., designated zones in the vicinity of a dumpsite. Figure 3 shows one of these incidents related to dumped munition.

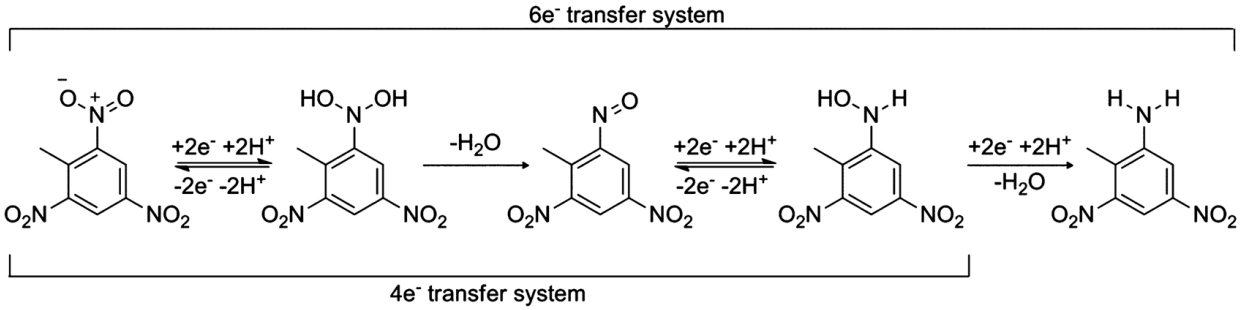
Figure 3. A lump of solidified sulfur mustard trawled up with fishes. As it contained explosives, it was emergency-relocated.[24]

2.2. TNT and its degradation products

2.2.1. Degradation

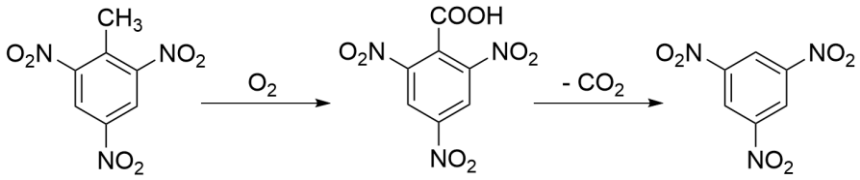
Microbial degradation is the primary natural process for removing organic pollutants, including explosives, from soil and sediment.[31] Many bacteria such as *Pseudomonas* and *Bacillus* have been documented as able to degrade TNT and its byproducts.[32–36] The type of microorganism and the culture conditions determine the main products and the degree of TNT degradation but the de-nitration pathway offers the highest advantage to microbial communities.[37] During bacterial metabolism of TNT, the nitro groups on the benzene ring are mainly converted into derivatives through three consecutive electron pair exchanges as shown in Scheme 1.[31,36] First, the reduction of one nitro group by nitroreductases proceeds via $2e^-/2H^+$ transfer to generate, with the elimination of a water molecule, the nitroso intermediate. This intermediate is easily reduced to the hydroxylamine intermediate through the same process. Finally, the hydroxylamine intermediate is reduced to the aromatic amine upon loss of a water molecule. Since the aminonitrotoluenes 4-amino-2,6-dinitrotoluene (4-ADNT) and 2-amino-4,6-

dinitrotoluene (2-ADNT) are the major byproducts of TNT environmental degradation, reductive de-nitration of TNT to produce 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) is limited.

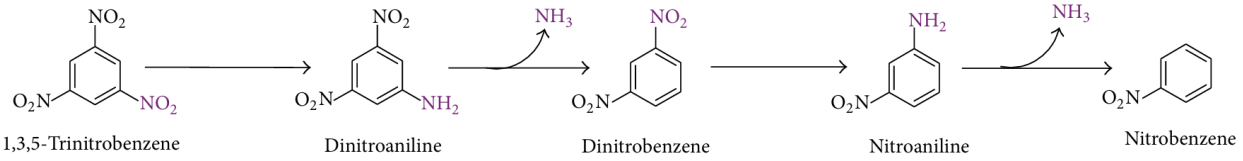


Scheme 1. Electrochemical reduction in aqueous media of the nitro group ortho to the methyl group in TNT resulting in 2-ADNT (reproduced with permission).[36]

Aerobic oxidation of the methyl group in the TNT molecule followed by decarboxylation forms the derivative trinitrobenzene (Scheme 2).[38,39] A bacteria community can further reduce trinitrobenzene (TNB) to dinitroaniline, which is eventually deaminated to dinitrobenzene and finally transformed to nitrobenzene (NB) through reduction and subsequent deamination (Scheme 3).[40]



Scheme 2. TNT oxidation and further decarboxylation to produce TNB.



Scheme 3. Degradation pathway of TNB.[40]

TNT and its degradation products can be detected using different analytical techniques, such as GC–MS/MS and liquid chromatography tandem mass spectrometry (LC–MS/MS), which will be discussed in more details in chapter 2.3.4.

2.2.2. Impact on biota

Strehse et al. (2017) showed that blue mussels *Mytilus edulis*, bivalves widely used as bioindicator, accumulate TNT and its main metabolites 2-ADNT and 4-ADNT when placed near explosive materials lying on the sea ground of the Kolberger Heide area (KH, former dumping site in the western Baltic Sea).[20] Out of eight mussels placed for 93 days on a bulk of explosive, TNT was found in six molluscs and both 2-ADNT and 4-ADNT in all of them, with concentrations in the range of ng/g mussel wet weight.

Studying the same dumping site, Beck et al. (2022) collected and investigated for munition compound (MC) contamination plankton, macroalgae, tunicate, sponge, mollusc, echinoderm, polychaete, anemone, crustacea, and fish. At least one MC was detected in more than 98% of the organisms collected, suggesting widespread exposure to MC contamination.[41] In plaice (*Pleuronectes platessa*) and flounder (*Paralichthys dentatus*), commercially fish species found in this region, TNT and 4-ADNT were detected in 17% and 33% of samples, respectively. Although concentrations of these compounds were extremely low, suggesting negligible carcinogenic risk due to TNT exposure from seafood consumption (according to Oral Cancer Slope Factor - CSF), health risk should not be completely excluded since the CSF estimation is based on limited data from non-human species.

Koske et al. (2020) evaluated the contamination status of dab (*Limanda limanda*) from the KH and three reference areas.[42] Bile samples were extracted and analyzed to verify the presence of TNT, RDX, cyclotetramethylene-tetranitramine (HMX) and four TNT degradation products (2-ADNT, 4-ADNT, 2,4-DNT and 2,5-DNT). 48% of samples collected from KH contained at least one target compound, making this study the earliest known evidence of explosives and their degradation products in dab. Among this 48%, 2-ADNT (12%, 1.60 ng/mL) and 4-ADNT (45%, 17.06 ng/mL) were more often detected and present at higher levels than TNT (2%, 0.06 ng/mL). As for the reference sites, only four samples out of 121 analyzed contained either HMX or 4-ADNT.

When assessing the environmental risks posed by sea-dumped chemical munitions, it is also important to consider climate change as this phenomenon can alter abiotic conditions and therefore influence munition shells corrosion, degradation rate and biota interaction

with MCs.[14] For instance, the increase in ocean salinity accelerates the corrosion rates of shipwrecks, which can result in the release of more toxic compounds into the environment.[14]

2.3. Analysis of organic explosives in environmental samples

Due to the low solubility of explosives in water (e.g., 0.013 g TNT/100 g water at 20°C),[16,17] studies on munition contamination in the Baltic Sea typically include sediment samples in addition to water samples. Prior to instrumental analysis, the target compounds must be extracted from the sample using an organic solvent that is preferably compatible with the analytical technique, thereby eliminating the need for additional steps such as solvent exchange. An extraction procedure, besides concentrating analytes, also aims to remove matrix interferents, one of the main challenges in environmental sample analysis. The most effective protocol for one analyte may be the least suitable for another, meaning that considerable effort is required to define the best overall procedure.

Environmental analysis require highly selective, sensitive and robust techniques because of the complex and heterogeneous nature of the matrices (e.g. soil, sediment, water) and the low concentrations (trace or ultra-trace levels) of the target analytes. To overcome these challenges, analytical methods such as LC–MS/MS and GC–MS/MS are commonly used and continuously optimized to ensure enhanced sensitivity, accuracy and reproducibility. In addition to the instrumental parameters that can be optimized, these techniques offer considerable flexibility through various configurations including different types of mass analyzers and ionization sources. This flexibility allows methods to be tailored to meet specific requirements, such as high-resolution screening.

2.3.1. Sample preparation

Published by the U.S. Environmental Protection Agency (EPA), the EPA Method 8330B (SW-846) is an analytical procedure for determining nitroaromatics, nitramines, and nitrate esters by high performance liquid chromatography (HPLC) in water, soil, and sediment matrix. This method is intended to guide laboratory personnel in developing their own Standard Operating Procedure (SOP). It includes the extraction of soil and sediment samples using acetonitrile (ACN) and an ultrasonic bath, or shaker, for 18 h.[43]

Dawidziuk et al. (2018) developed and validated three methods of sample preparation for identification and quantification of TNT and its degradation compounds (TNB, 1,3-DNB, 2,4-DNT, 2,6-DNT, 4-NT and 2,4-DNA) in bottom sediment samples from the Baltic Sea: SLE, Soxhlet extraction (SE) and accelerated solvent extraction (ASE).[12] Extractions were carried out using chloroform ($CHCl_3$). SE was the best extraction method evaluated, with recoveries ranging from 80.7% to 98.2% for all compounds, but it was the most time-consuming technique, requiring up to 24 hours. After optimization of four parameters (extraction temperature, static time, flush volume, and extraction cycles), ASE led to recoveries from 71.8% to 87.7%. Although faster than SE, ASE uses expensive equipment not commonly found in every laboratory. Achieving analyte recoveries from 64.6% to 91.8% with triple extraction and 2.5 minutes, SLE was the recommended method for the investigation of explosives in bottom sediments due to the short processing time and lack of requirement for complicated equipment.

SLE was also employed by Gordon et al. (2018) for the study of TNT degradation kinetics in simulated sediment samples from the Baltic Sea.[16] To monitor transformations of TNT in the environmental samples and further determine degradation kinetic curves and identify the reaction products, the most favorable sample preparation procedure had to first be defined. For this, two solvents, acetone and $CHCl_3$, and two drying methods, magnesium sulfate ($MgSO_4$) and nitrogen gas, were investigated. Highest TNT recoveries were observed for $CHCl_3$, and the use of $MgSO_4$ and nitrogen gas stream provided comparable recoveries (90.5% and 89.8%, respectively). Extractions with acetone provided less than 65% recovery. Further sediment analysis was performed with $CHCl_3$ and $MgSO_4$ given its low cost and high efficiency.

To study sediment samples from the Baltic Sea floor, Nawała et al. (2020) defined two different sample preparation protocols depending on the analysis technique.[18] For GC analysis, samples were extracted with $CHCl_3$, and extracts were dried using anhydrous $MgSO_4$ and for LC analysis, extraction was done with acetone without a drying agent. Spiking experiments to study recoveries were not conducted. Analysis of sediment samples allowed the identification of the following compounds: 2,4-DNT, 2-ADNT, 4-ADNT, 1,3-DNB, 3,5-DNT, TNT, and RDX. The last two explosives were also detected in large quantities in samples of explosives excavated from the seabed, along with aluminum

powder, indicating that the material could be a torpex (explosive containing RDX, TNT and aluminum widely used in World War II).

Mu et al. (2012) developed an analytical method for quantitative determination of nitroguanidine and 2,4-dinitroanisole in soil, tap water, and river water by using ultrafast LC–MS/MS but TNT, RDX, HMX, PETN and 2-ADNT were also included to make the method more comprehensive.[44] Unlike the previously mentioned studies, in this work soil samples were extracted with pure methanol (MeOH) and MeOH–water (50:50, v/v). Recovery of TNT and 2-ADNT in spiked soil samples were 70.2% and 78.5%, respectively when extraction was carried out with MeOH-water and 88.1% and 97.7%, respectively when pure MeOH was used instead.

Ideally, recovery studies should use compound concentration closely aligned with those expected in real samples, simulating actual conditions. However, because explosives are typically present in trace amounts in the environment, tests are usually performed at higher spiking levels (ppm) to minimize matrix effects and more effectively optimize extraction parameters. For instance, Gordon et al. (2018) spiked sediment samples with TNT in two distinct levels: 1 mg/g (TNT/sediment) for the optimization of the sample preparation procedure and 10 mg/g for the TNT transformation studies.[16]

An overview of the literature discussed in this chapter is presented in Table 1.

Table 1. Summary of literature on organic explosives extraction from environmental samples.

Ref.	Compounds	Sample type	Spiking (TNT/g soil or sediment)	Extraction solvent	Instrument
[44]	HMX, RDX, TNT, 2-ADNT, PETN	Soil	1 ng/g	MeOH, 50:50 MeOH-water (soil)	LC–MS/MS
[12]	TNT, TNB, 1,3-DNB, 2,4-DNT, 2,6-DNT, 4-NT, 2,4-DNA	Marine sediment	1.25 mg/g	$CHCl_3$	GC–MS/MS
[16]	4-NT, 1,3-DNB, 2,6-DNT, 2,4-DNT, TNB, TNT, 2-ADNT, 4-ADNT, 2,4-DNA	Marine sediment	1 mg/g	Acetone, $CHCl_3$	GC–MS/MS, LC–MS/MS
[18]	TNT, RDX, 2-ADNT, 4-ADNT, 2,4-DNT, 1,3-DNB, 3,5-DNT, TNB	Marine sediment	-	Acetone, $CHCl_3$	GC–MS/MS, LC–HRMS, NMR

2.3.1.1. Clean-up methods

As marine sediment and seawater are complex matrices, removal of potential interferents during sample preparation is essential to obtain an accurate and unbiased validation process for the determination of target compounds.[45] Although extraction into organic solvents eliminates water-soluble contaminants, countless organic-soluble compounds remain in the samples. These matrix compounds might lead to instrumental issues such as signal suppression/enhancement, baseline instability, and ghost peaks.[19] Matrix effects can be mitigated through several clean-up procedures such as SPE, solvent-assisted dispersive solid-phase extraction (SADSPE), and solid-phase microextraction (SPME).[46] Due to its simplicity and low solvent consumption, SPE is the most employed pre-concentration technique for the analysis of organic explosives.[17,46–48]

2.3.1.1.1. Solid-phase extraction (SPE)

A typical SPE procedure consists of four steps: (i) conditioning the solid-phase: solvent or mixture of solvents activate the sorbent, (ii) sample loading: introduction of sample solution into the SPE material, (iii) rinsing/washing: unwanted matrix components are washed away, and (iv) elution of analytes: interaction between solid-phase and analytes is disrupted and eluate with target species is collected.[49,50] These basic SPE steps are shown in Figure 4.

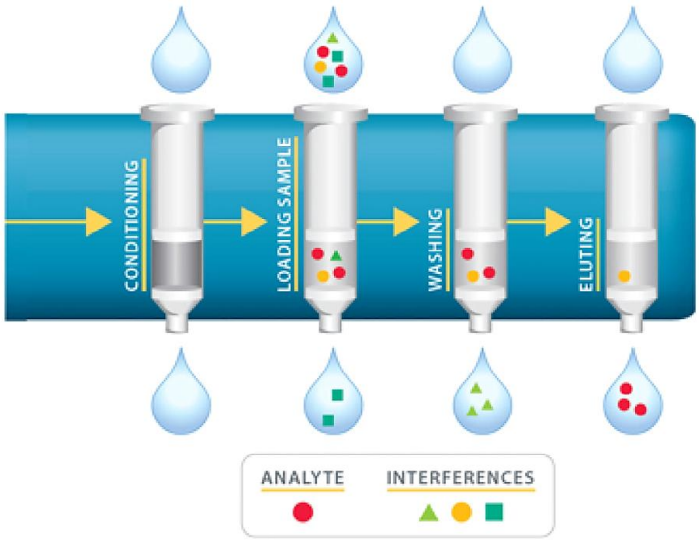


Figure 4. SPE scheme (reproduced with permission).[49]

A wide range of SPE formats and sorbent materials are commercially available. According to the literature, alkyl-bonded silicas and copolymers are the most used sorbent materials for reversed-phase separations.[49] Among this class, hydrophilic-lipophilic balanced polymers are the most popular choice. This type of sorbent consists of a balance ratio of two monomers: lipophilic divinylbenzene (DVB) and hydrophilic N-vinylpyrrolidone, making it an excellent choice for polar and non-polar compounds.

Rapp-Wright et al. (2017) evaluated 34 SPE-sorbents for the analysis of organic explosives in wastewater prior to analysis with liquid chromatography-high resolution mass spectrometry (LC–HRMS).[46] Oasis HLB was the best performing cartridge with highest and less variable recoveries but overall, promising results were found for 'mixed-mode' co-polymeric styrene-divinylbenzene (PS-DVB) sorbents. This HLB-type cartridge was also investigated by Detata et al. (2013) for the extraction of explosive residues from hand swabs and postblast debris.[51] In this work, separation and detection of analytes were conducted by liquid chromatography-ultraviolet (LC–UV) and different clean-up procedures were evaluated. In addition to Oasis HLB, two SPE-cartridges were investigated, one containing polyvinylpyrrolidone–divinylbenzene copolymer (Porapak RDX) and the other silica gel (Supelclean LC-Si). The HLB-cartridge outperformed the other two with an average of analyte recoveries of 94%.

To recover trace levels of nitro-organic explosives in soil, Thomas et al. (2018) compared the performance of three copolymeric SPE cartridges for use with explosives using methods from the literature.[19] Five factors were assessed: supply cost, method complexity, explosives recovery, processing time and matrix rejection. Extracted fortified soil samples were processed with Bond Elut NEXUS, Empore SDB-XC, and Oasis HLB. Oasis HLB cartridges provided the highest initial average recovery (35%) but had the longest processing times (1.5–3 h), which could be attributed to the small particle size and pore size of the sorbent (30 μm and 80 Å) and presence of particulates in the extract. After modification of the elution solvent for compatibility with GC analysis (from 90:10 MeOH-water to 100% MeOH), the SDB-XC cartridges provided similar average recoveries as the HLB cartridges but had faster processing times (1–2 h). This cartridge had the highest cost overall of the tested cartridges and was not further investigated. SPE with NEXUS cartridges had the fastest processing time (less than an hour) but low recoveries, with an

average of 15%. After dilution of the acetone extracts in water, recoveries for all explosives increased, averaging between 34 and 45%. Due to its low cost, fast processing time and larger particle and pore size, method development was continued with Bond Elut NEXUS.

Gledhill et al. (2019) used CHROMABOND Easy polystyrene-divinylbenzene-copolymer SPE cartridges to first pre-concentrate and then quantify munition compounds in seawater samples from the KH.[17] Spiked samples were preconcentrated in the SPE columns after a conditioning step with ACN followed by water. Then, the columns were washed with water and eluted with ACN to recover the analytes. More than 50% recovery was obtained for TNT, TNB, 2,4-DNT, 2,6-DNT, 2-ADNT, and 4-ADNT. Later, this developed method was adapted and applied by Bünning et al. (2021) to determine nitroaromatic explosives in marine sediment samples by GC–MS/MS.[52] Lower limits of detection were obtained when SLE of 2 g of sediment with ACN was replaced by SPE of 100 g of wet sediment mixed with doubly distilled water.

2.3.1.1.2. Solvent-assisted dispersive solid-phase extraction (SADSPE)

Another clean-up approach for environmental aqueous samples detailed in the literature is SADSPE. This method is based on the injection of milligram amounts of a sorbent dissolved in a water-miscible solvent to an aqueous sample. The dispersion of fine particles of the sorbent in the bulk aqueous sample results in a cloudy solution. Phase separation is achieved by centrifugation. After, the sedimented phase is collected, dissolved in a suitable solvent and analytes are determined using an instrumental technique (Figure 5).[53]

Setayeshfar et al. (2024) used MeOH containing benzophenone to extract TNT, RDX and HMX from aqueous samples and subsequently analyzed them by high performance liquid chromatography-ultraviolet (HPLC–UV).[54] After optimizations, the final method included 10-30 seconds extraction with MeOH with sorbent to solvent ratio of 4% (w/v), no pH-adjustment and 4 min centrifugation. The analysis of lake and river water samples with the optimized SADSPE procedure resulted in relative recoveries between 96% and 106% and relative standard deviations (n = 3) in the range of 2–3.5%, which demonstrates

the high efficiency of the method for measuring explosive compounds in different water samples.

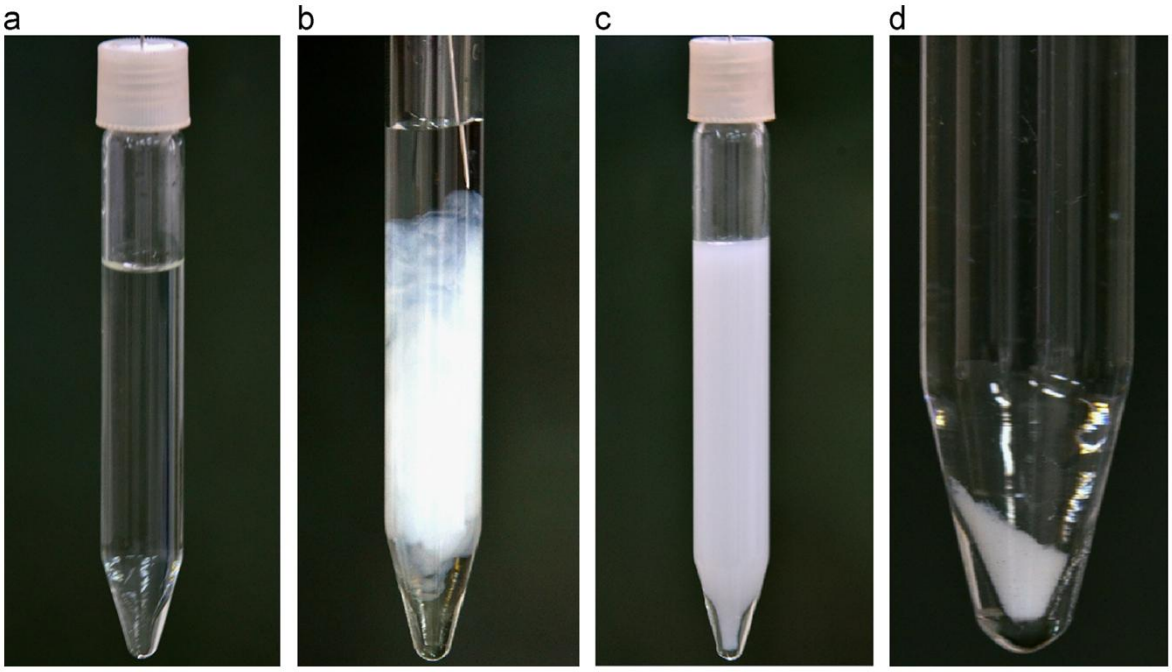


Figure 5. Photo of SADSPE steps: (a) sample solution, (b) injection of mixture containing disperser solvent and sorbent to the sample, (c) end of injection (cloudy state), and (d) enlarged view of sedimented phase after centrifugation (reproduced with permission).[53]

2.3.1.1.3. Solid-phase microextraction (SPME)

SPME is a sample preparation technique that uses fiber sampler devices coated with an adsorbent material to extract semi-volatile/volatile analytes from complex matrices.[50] This procedure can be performed using either direct immersion, in which the fiber is placed in direct contact with the sample solution to extract the analytes, or headspace mode, where the fiber is positioned above the solution to remove the volatile compounds (Figure 6). After extraction, the fiber is retracted to the needle to avoid contamination and then inserted into the injection port of the analytical instrument, such as GC–MS and LC–MS, for desorption and subsequent analysis.

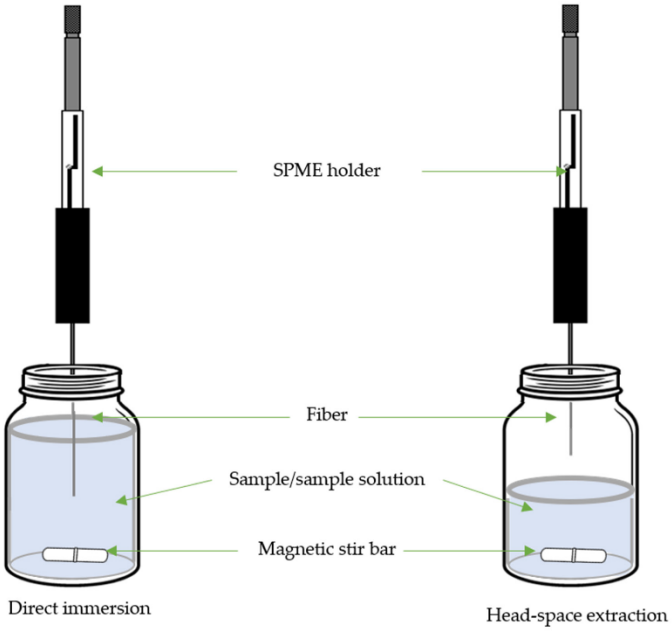


Figure 6. SPME scheme.[55]

Although recent literature on extraction/clean-up of explosives from environmental samples is more focused on SPE, due to its higher sensitivity (exhaustive technique), several studies exploring SPME were published in the early 2000s.[56] Monteil-Rivera et al. (2004) used SPME to determine HMX, RDX, TNB, 1,3-DNB, tetryl, 3,4-DNT, TNT, 4-ADNT, and 2,4-DNT in environmental water samples.[57] Many parameters were optimized (e.g. adsorption and desorption time) to obtain reproducible data with satisfactory accuracy and three fiber coatings were tested: polyacrylate (PA), Carbowax/templated resin (CW/TPR) and polydimethylsiloxane/divinylbenzene (PDMS-DVB) fiber. Later, SPME with CW/TPR was compared to SPE using Porapak Rdx Sep-Pak cartridge. HMX, RDX and 4-ADNT were identified in the ppb range in groundwater samples by the two methods with an excellent agreement. However, detection limits for SPME were 10 times higher than the detection limits obtained using SPE, evidencing its lower sensitivity.

Table 2 summarizes the literature covered in this chapter about clean-up procedures for the identification of explosives in environmental samples.

Table 2. Summary of references on clean-up approaches for organic explosives analysis in environmental samples.

Ref.	Compounds	Sample type	Clean-up method	SPE procedure[1]	Instrument
52	1,3-DNB, 2,4-DNT, 4-ADNT, TNT, 2-ADNT	Seawater, sediment	SPE	E: ACN	GC–MS/MS
46	HMTD, R-salt, HMX, EGDN, RDX, TATP, 3,4-DNT, 2-NT, 4-NT, 2,6-DNT, 2,4-DNT, Tetryl, TNT, PETN, NB, NG, 3-NT, ETN	Wastewater	SPE	C: MeOH, water W: water E: ACN[2]	LC–HRMS
17	HMX, RDX, NG, PETN, Tetryl, TNB, 1,3-DNB, NB, 3,5-DNA, TNT, 2,6-DNT, 2,4-DNT, 4-NT, 2-NT, 3-NT, 4-ADNT, 2-ADNT	Seawater	SPE	C: ACN, water W: water E: ACN	UHPLC–MS
19	DMDNB, DNT, HMX, 4-NT, NG, PETN, RDX, Tetryl, TNT, ETN, EGDN R-salt	Soil	SPE	C: MeOH, water W: 50:50 MeOH-water E: MeOH[2]	GC–ECD
54	HMX, RDX, TNT	Lake/river water	SADSPE	-	HPLC–UV
57	HMX, RDX, TNB, 1,3-DNB, 3,4-DNT, TNT, 4-ADNT, 2,4-DNT, Tetryl	Seawater, groundwater	SPME	-	HPLC–UV

1 C: conditioning; W: wash; E: elution.
2 SPE method for Oasis HLB cartridge.

2.3.2. Analytical methods

Routinely applied analytical procedures for identifying contaminants in environmental samples typically involve liquid or gas chromatography, coupled with varying detection systems such as mass spectrometry (MS) or ultraviolet (UV) spectroscopy.[12,16,18] These techniques have distinct advantages and disadvantages, and their effectiveness as analytical methods depends not only on their inherent features such as selectivity and sensitivity, but also on their compatibility with the target analytes and the complexity of the sample matrix. Therefore, selecting the most suitable analytical approach for an intended purpose can significantly impact on the quality and relevance of the work conducted, and lead to more reliable and accurate results.

2.3.2.1. Gas chromatography (GC)

GC is a powerful separation technique well-suited for volatile and semi-volatile compounds that exhibit sufficient thermal stability. To extend its applicability, derivatization reactions can be applied to chemically modify analytes and obtain more volatile and thermally stable derivatives. As with other instrumental techniques, GC analysis performance is highly dependent on the precise optimization of key operational parameters, including oven temperature program, injection mode and type and flow rate of the carrier gas. Each of these variables can significantly influence the chromatographic resolution, analyte recovery, and overall sensitivity of the method, which is further affected by the choice of detector. GC coupled with electron capture detector (ECD) and tandem mass spectrometry (MS/MS) will be briefly reviewed in this chapter.

2.3.2.1.1. Gas chromatography with electron capture detection (GC–ECD)

ECD is a highly selective and sensitive detector used in gas chromatography that measures compounds based on their ability to capture electrons from the constant electron current, making it particularly effective for detecting analytes containing electronegative atoms such as nitroaromatic compounds.

GC–ECD was selected by Thomas et. al (2018) for screening 12 nitro-organic explosive compounds in soil samples.[19] Detection was carried out based on retention time matching, where an analyte was positively identified if its retention time deviated no more than ± 0.01 min from that of the corresponding reference standard. This reliance on retention time represents a major disadvantage of GC–ECD, particularly in the analysis of complex matrix environmental samples where retention time shifts are frequently observed. As part of the method validation, an interference study revealed that among 13 soil samples analyzed, five interfering peaks were detected, corresponding to a 3.2% false positive rate for the validated method. This highlights another drawback of this technique: its inability to distinguish co-eluting electron-capturing interferents from target analytes.

2.3.2.1.2. Gas chromatography-tandem mass spectrometry (GC–MS/MS)

Electron ionization (EI) typically at 70 eV, is the most popular ionization technique used in GC–MS and GC–MS/MS analysis, offering highly reproducible and extensively fragmented mass spectra. The fragmentation pattern produced by EI serves as a unique "fingerprint" that can be compared against hundreds of thousands of EI-mass spectra compiled in various spectral libraries for molecular identification.[58]

While this library-based identification approach is well suited for full scan analysis, it becomes inapplicable when tandem mass spectrometry is employed, particularly in Multiple Reaction Monitoring (MRM) mode, as full fragmentation spectra are not generated.

MRM is a targeted MS/MS technique that allows the detection and quantitation of several pre-defined compounds in complex matrices.[59] Unlike in full scan MS or MS/MS, where the mass detector continuously scans all ionized compounds and fragments, in MRM mode, specific precursor-product ion pairs are selected and monitored throughout the entire chromatographic run or only around the expected elution time.[12] In the latter approach, known as scheduled MRM, method sensitivity is enhanced by reducing the number of ions monitored at the same time, allowing for more scans per transition and consequently better peak shapes. Overall, MRM offers enhanced signal-to-noise ratios (S/N), greater selectivity, and improved precision in quantitative analysis.

Gordon et al. (2018), Dawidziuk et al. (2018) and Nawała et al. (2020) employed GC–MS/MS in MRM mode for the analysis of explosives in marine sediment samples from the Baltic Sea.[12,16,18] With the exception of TNB, which was only detected using LC–HRMS in the study by Nawała et al. (2020), the developed and optimized GC–MS/MS methods enabled the determination of TNT and its main degradation products.

2.3.2.2. Liquid chromatography (LC)

LC is applicable to a broad range of compounds, including polar, non-volatile, and thermally unstable analytes. In addition, it is suitable for direct analysis of aqueous samples, without the need for solvent exchange–step required in GC analysis. Method

performance relies on the optimization of several parameters, such as mobile phase composition and elution mode. LC can be combined with various detector types, each tailored to specific analytical needs. In this chapter, LC coupled with HRMS, UV and MS/MS will be discussed.

2.3.2.2.1. Liquid chromatography with high resolution mass spectrometry (LC–HRMS)

As mentioned previously, LC analysis has the main advantage that analytes are not exposed to high temperatures as in GC, which is especially significant when working with thermally labile compounds.[16] RDX, for instance, is relatively unstable at high temperatures and therefore, GC analysis is challenging (e.g. injector temperature must be optimized) and should be avoided especially when focusing on quantitation studies. This is evidenced in the studied conducted by Nawała et al. (2020), where the quantitation errors for RDX in explosive samples from the Baltic Sea were about 15% by GC–MS/MS and 2% when liquid chromatography with high resolution mass spectrometry (LC–HRMS) was employed.[18] These results indicated that the LC–HRMS analysis provided more reliable quantitation results for RDX. Although LC–HRMS provides high-resolution and accurate mass measurements, its operational complexity, limited linear dynamic range, higher cost and demanding data processing make it less suitable for targeted analysis compared to LC–MS/MS.[60,61]

2.3.2.2.2. High performance liquid chromatography-ultraviolet (HPLC–UV)

HPLC–UV, robust analytical technique with operational simplicity, was selected by Setayeshfar et al. (2024) to detect HMX, RDX, and TNT in extracted aqueous samples.[54] A spiking study with real samples showed that matrix compounds did not significantly interfere in the target analytes detection, which was mainly attributed to the clean-up technique carried out before analysis. Although effective for the three selected compounds, the developed method may not be suitable for targeted trace analysis considering its limited sensitivity and poor selectivity.[60]

2.3.2.2.3. Liquid chromatography-tandem mass spectrometry (LC–MS/MS)

LC–MS/MS is an advanced analytical tool ideal for quantitative and qualitative detection of explosives and its metabolites at trace level. An advantageous feature in LC–MS/MS is that soft ionization techniques are frequently used; most commonly electrospray ionization (ESI). These methods employ low-energy ionization processes that minimize fragmentation, preserve molecular ions, and thereby facilitate spectral interpretation.

Kober et al. (2019) extracted soil samples from a former TNT production site in Germany based on the EPA 8330B procedure and analyzed the extracts by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).[62] TNT and ADNTs were found in the samples but the method was not able to distinguish the regioisomers 2-ADNT and 4-ADNT, since MALDI provides minimal fragmentation and these compounds have identical molecular masses. Additionally, in MALDI, analytes are co-crystallized with a matrix on a target plate and ionized using a laser, which makes this technique incompatible with online LC, thereby preventing continuous separation of analytes. However, with an ESI-LC–MS/MS analysis conducted as part of the study, 2-ADNT and 4-ADNT were successfully detected and quantified in the samples owing to their different retention times and fragmentation patterns.

2.4. Conclusion

The literature reviewed in this chapter highlights the growing concern regarding sea-dumped munitions, as evidenced by the establishment of several international initiatives dedicated to monitoring dumping sites, developing remediation strategies, and analytical methods to evaluate munition contamination levels in the environment. TNT microbial degradation was assessed as well as its impact on marine biota. Several studies on analysis of organic explosives in environmental samples were discussed, with particular focus on the different sample preparation approaches implemented, including extraction procedures and clean-up methods, and the instrumental techniques used, with emphasis on gas and liquid chromatography-based methods. Their strengths and limitations were evaluated, particularly regarding sensitivity, selectivity, operational complexity, and suitability for the target analytes.

3 Experimental Section

Numerous studies have focused on the identification and quantitation of TNT and its degradation products in environmental samples, but to our knowledge, no study has yet comprehensively addressed all the selected compounds proposed for investigation in this thesis in marine sediment samples by GC–MS/MS.

This section outlines the materials, sample preparation procedures, and analytical method employed throughout the experimental work of this thesis. In subsequent subsection division, it provides a detailed description of the extraction protocols, proposed clean-up procedures, optimization of the MRM method for the GC–MS/MS analysis and recovery studies performed. Lastly, this chapter presents a discussion of the experimental findings, comparing outcomes obtained with the reference method and the optimized protocol, highlighting improvements in analytical performance. Limitations encountered during the study and suggestions for future research are addressed as well at the end of the section.

3.1. Materials and chemicals

HPLC-grade ethyl acetate (≥ 99.7%, Honeywell), HPLC-grade dichloromethane (99.8%, Fischer Chemicals), HPLC-grade chloroform (≥ 99.8%, Fischer Chemicals), LC-MS grade methanol (≥ 99.9%, Fischer Chemicals), GC-grade dichloromethane (Supelco) and ultrapure water (18.2 MΩ cm) produced with Millipore Direct-Q®3 UV system were the solvents used throughout sample preparation and analysis.

All standard solutions were prepared either directly from commercial reference materials (>=99.8%) or by diluting pre-made stock solutions (prepared in DCM or ACN) from reference chemicals with purities greater than 99%.

2-nitrotoluene (2-NT), 3-nitrotoluene (3-NT), 4-nitrotoluene (4-NT) and hexachlorobenzene (HCB) reference chemicals were obtained from Sigma; 2,4-DNT and TNT from Cerilliant; 2-ADNT, 4-ADNT, 2,6-DNT and NB from Supelco; TNB from Accustandard; and deuterated 4-nitrotoluene (4-NT d7) from CDN Isotopes.

$MgSO_4$ (62-70%) and Na_2SO_4 (99.6%) were both obtained from Fischer Chemicals, dried before used and stored in a desiccator.

CHROMABOND Easy (polystyrene-divinylbenzene-copolymer, 6 mL/200 mg), Oasis HLB (polyvinylpyrrolidone-divinylbenzene copolymer, 6 mL/200 mg) and Strata Si-1 (silica, 6 mL/500 mg) were purchased from Macherey-Nagel, Waters Corporation and Phenomenex, respectively.

3.2. Sampling

The marine sediment utilized in this study was collected in April 2021 by the Institute of Oceanology Polish Academy of Sciences (IO PAN) from the known dumping area of Bornholm (55.19568, 15.38022) from 93 meters depth.[3] The sediment was stored at 4°C until the start of the experiment. Figure 7 shows the Bornholm dumping site located in the Baltic Sea.

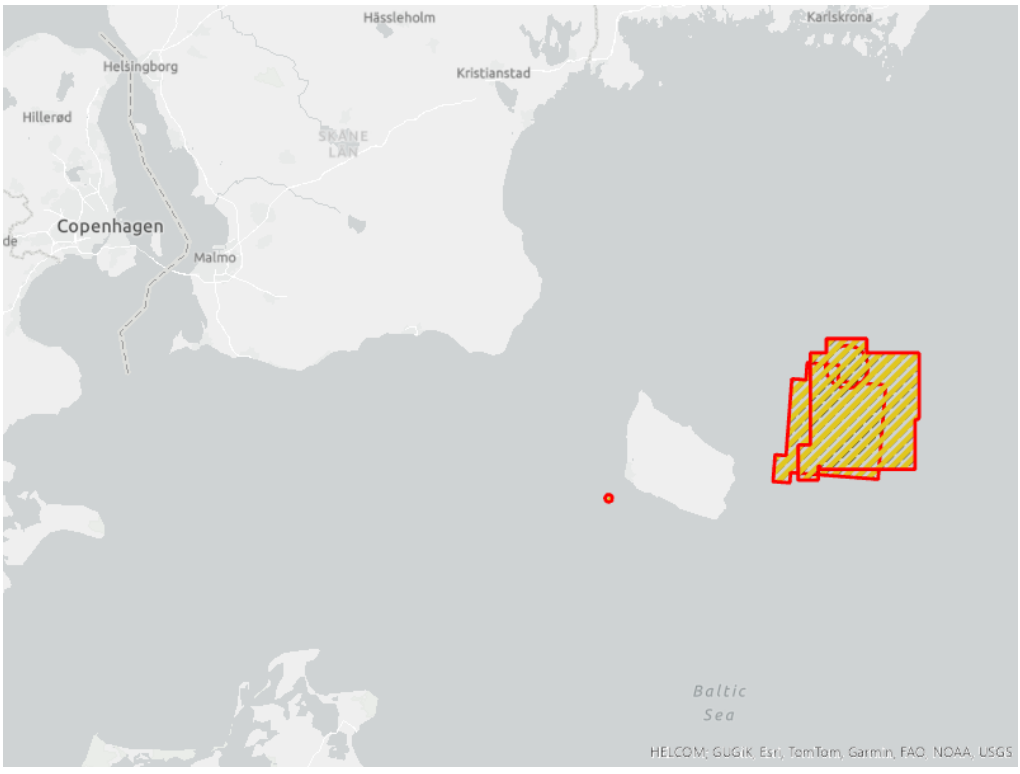
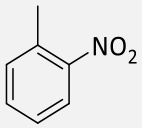
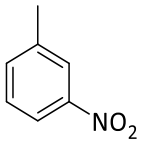
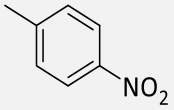
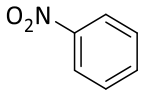
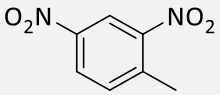
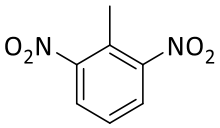
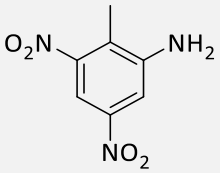
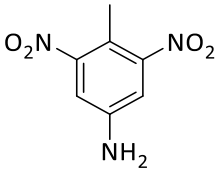


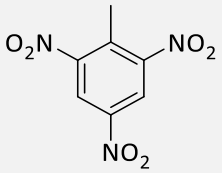
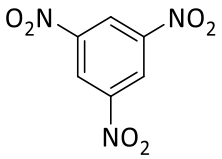
Figure 7. Bornholm dumping site (HELCOM Data and Map Data Service).

3.3. Preparation of samples for the optimization studies

The target chemicals for this study are listed in Table 3.

Table 3. TNT and its degradation products discussed in this thesis.

Compound	Acronym	Structure	Spiking level (µg/mL)	Vapor pressure (atm at 25 °C)	Ref.
2-Nitrotoluene	2-NT		20	1.89×10^{-4}	63
3-Nitrotoluene	3-NT		20	~ 1.18×10^{-4}	64
4-Nitrotoluene	4-NT		20	6.43×10^{-5}	63
Nitrobenzene	NB		15	1.97×10^{-4}	65
2,4-Dinitrotoluene	2,4-DNT		5	4.11×10^{-7}	66
2,6-Dinitrotoluene	2,6-DNT		5	8.93×10^{-7}	66
2-Amino-4,6-dinitrotoluene	2-ADNT		5	6.05×10^{-8}	67
4-Amino-2,6-dinitrotoluene	4-ADNT		5	1.05×10^{-8}	68

Compound	Acronym	Structure	Spiking level (µg/mL)	Vapor pressure (atm at 25 °C)	Ref.
2,4,6-Trinitrotoluene	TNT		5	1.05×10^{-8}	60
1,3,5-Trinitrobenzene	TNB		5	2.00×10^{-8}	60

3.3.1. Standard solutions

Intermediate standard solutions at appropriate concentrations were prepared from pre-made stock solutions of the target compounds. Aliquots of the intermediate solutions were used to make the standard explosive mixture solution to spike the sediment portions, and the concentration levels of the standards are presented in Table 3. The less volatile compounds were spiked with higher concentrations to allow appropriate extraction and detection. All solutions were prepared in dichloromethane (DCM).

As for the internal standards (c = 1 µg/mL), HCB was only prepared in DCM, since this compound is the internal standard (ISTD) used in the reference method, and the 4-NT d7 solutions were prepared in, besides DCM, ethyl acetate (EtOAc), 50:50 ethyl acetate-dichloromethane (EtOAc-DCM) and $CHCl_3$ to be evaluated as an alternative ISTD.

The prepared standard solutions were analyzed with GC–MS/MS in MRM mode to verify the quality of the preparations. More detailed description of the MRM method is provided in chapter 3.5.1.

3.3.2. Spiking

For the spiking procedure, the sediment sample was taken from the refrigerator and placed inside a fume hood to reach room temperature. After the temperature of the sample was equilibrated, 10±1 g-portions of sediment were weighed into Falcon tubes and spiked with 50 µL of the standard explosive mixture solution containing the target compounds.

This resulted in analyte concentrations in the sediment of approximately 25 ng/g, 75 ng/g, and 100 ng/g, depending on the spiking level. Blank samples, containing only sediment and no added analytes, were also prepared. Following homogenization, the samples were stored in the refrigerator for approximately 24 hours. Next, the spiked sediment portions and blanks were extracted according to the Recommended Operating Procedures for CWC-related Analysis, Chapter IX.B. "Analysis of explosives in sediment and soil".[69] Any extractions conducted following this procedure will be referred to as 'solvent + ROP'.

3.3.3. Extraction

The spiked portions and blanks were centrifuged for 3 minutes at 1000 x g using a Heraeus Multifuge X1R centrifuge (Thermo Scientific), and excess pore water was collected into a separate Falcon tube. This process was carried out twice. The centrifuged sediments were then extracted with 10 mL of GC-MS grade dichloromethane by shaking for 10 minutes (RT, 1000 rpm) using a Multi Reax shaker (Heldolph). Following this, the samples were again centrifuged, and the organic layer decanted through filter paper (Whatman 1PS) into a 20 mL volumetric flask. Extraction was repeated with another 10 mL-portion of clean solvent. The organic layers from the two extractions were combined and adjusted to a final volume of 20 mL. The dichloromethane extracts were transferred to EPA vials, dried with approximately 1 g of reagent grade anhydrous sodium sulfate (Na_2SO_4), and left to stand overnight in the refrigerator.

For the GC–MS/MS analysis, 10 mL-portions of the extracts were evaporated to near dryness at 45°C under nitrogen flow using a TurboVap LV Evaporation System (Caliper Life Sciences), adjusted to a final volume of 1 mL and filtrated using a Millex Samplicity 0.45 µm filter. Next, a 200 µL aliquot of the concentrated extract was diluted to 210 µL by the addition of 10 µL of 1 µg/mL HCB and analyzed as soon as possible. A schematic presentation of the sample preparation procedure is presented in Figure 8.

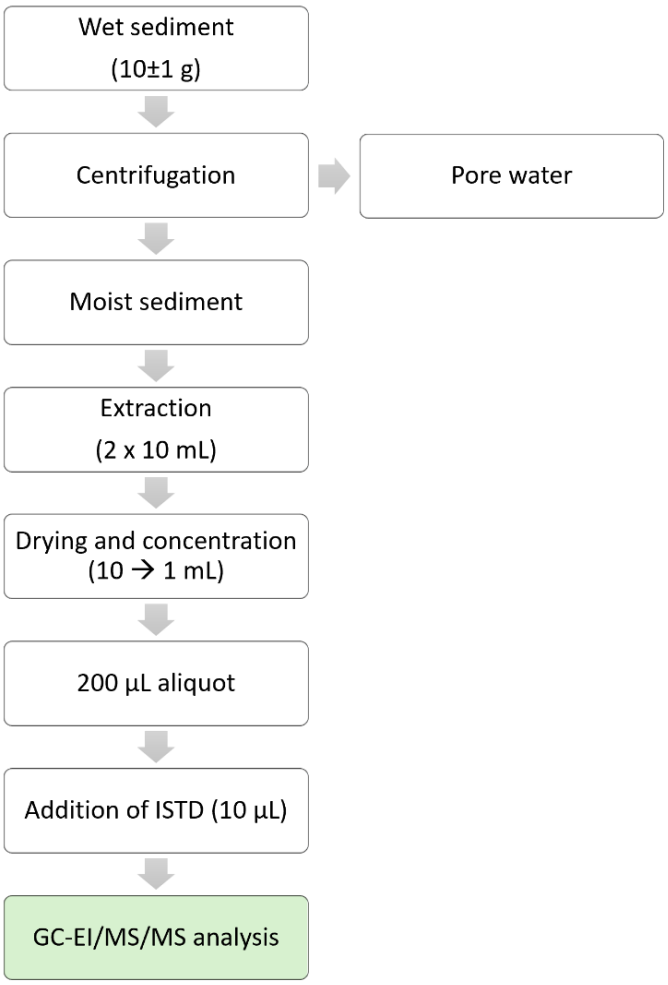


Figure 8. Sample preparation flow-chart for the sediment samples.

3.4. Optimization

The optimization study was divided into two parts: optimization of extraction method and development of a SPE clean-up procedure.

3.4.1. Optimization of sample preparation

3.4.1.1. Extraction

The parameters selected for modification in the extraction method described in section 3.3.3. include centrifugation conditions, shaking speed, drying agent, drying conditions, filter pore size, and internal standard. Table 4 summarizes the parameters studied and their modifications.

Table 4. Parameters investigated for the optimization of the extraction procedure.

Parameters	ROP	Optimization
Centrifugation	3 min, 1000 x g	5 min, 3000 x g
Shaking	10 min, 1000 rpm	10 min, 2000 rpm
Drying salt	Na_2SO_4	$MgSO_4$
Drying conditions	Refrigerator	Ambient temperature
Filter pore size	0.45 µm	0.2 µm
Internal standard	HCB	4-NT d7

With the optimized conditions, three different extraction solvents/mixed solvents were tested and compared to DCM: EtOAc, $CHCl_3$ and 50:50 (v/v) EtOAc-DCM. These solvents were selected based on their immiscibility with water, extraction ability, polarity, compatibility with GC systems and published studies.

DCM is considered a versatile solvent because of its moderate polarity and good interaction with hydrophobic compounds (dielectric constant 9.1 and dipole moment 1.6).[70] Another advantage is its high volatility, meaning that evaporation steps are faster than with less volatile solvents – on the downside, this brings more safety concerns.

Despite being more toxic than DCM, $CHCl_3$ was chosen due to its non-polar properties (dielectric constant 4.8 and dipole moment 1.04 D) and its use in previous extraction studies.[70]

EtOAc, a moderately polar green solvent commonly used in extractions and less harmful than chlorinated solvents, was tested as a potential substitute for DCM.[71] A mixture containing equal parts of EtOAc and DCM was also tested, taking advantage of the different polarity and solubility properties of each solvent and to reduce the volume of DCM used in the extraction.

Although acetone and ACN are also commonly employed in extraction studies of organic explosives from environmental samples, these two solvents were not closely investigated due to their miscibility with water, which could adversely affect the GC system in the long term.

3.4.1.2. Clean-up

All cartridges that were evaluated in the clean-up optimization study are presented in Table 5. SPE experiments were conducted with EtOAc and $CHCl_3$ dried extracts. The 20 mL-extract was divided into two 10 mL-portions for the SPE: one portion for experiments with Oasis HLB and the other with CHROMABOND Easy. Extractions were done in duplicate. To wash out impurities retained to the sorbent phase, four different washing methods were evaluated by varying MeOH content in water. Elution was carried out using EtOAc. Eluate was filtered through Whatman 1PS for water removal, concentrated to 1 mL, filtered with 0.2 µm filter and an aliquot with 4-NT d7 was prepared for analysis. Table 6 details the evaluated SPE protocols.

A second experiment, modeled after Thomas et al. (2018), was conducted with an initial solvent exchange step and using the Oasis HLB cartridge.[19] EtOAc extracts (n = 2) were evaporated to dryness, reconstituted in 4 mL of acetone and diluted to 50 mL with water. Elution was done with MeOH, and eluate was evaporated to dryness and reconstituted in EtOAc. This last step was performed to avoid poor chromatographic behavior (e.g. peak splitting) generally observed when MeOH is used in GC analysis and to prevent column damage caused by water present in the extract (since MeOH and water are miscible).

Lastly, Strata Si-1 was investigated. This normal-phase column was evaluated with $CHCl_3$ extracts (n = 2), following manufacturer instructions. MeOH and $CHCl_3$ were used to condition the cartridge. After sample loading, impurities were washed out with $CHCl_3$, and elution was carried out with MeOH. Solvent exchange was performed as detailed in the last paragraph.

Table 5. SPE cartridges used in this work.

Cartridge	Composition	Packing	Particle size	Pore size	Cartridge volume
Oasis HLB	Polyvinylpyrrolidone–divinylbenzene copolymer	200 mg	30 µm	80 Å	6 cc
CHROMABOND Easy	Polystyrene-divinylbenzene copolymer	200 mg	95 µm	50 Å	6 cc
Strata Si-1	Silica	500 mg	55 µm	70 Å	6 cc

Table 6. Solid-phase extraction protocols.

Step	Oasis[a] and CHROMABOND[b]	Oasis[a]	Strata Si-1[b]
Conditioning	2 × 5 mL MeOH 5 mL water	2 × 5 mL MeOH 5 mL water	5 mL MeOH 5 mL $CHCl_3$
Sample load	10 mL EtOAc extract	50 mL acetone extract diluted with water	10 mL $CHCl_3$ extract
Wash	5 mL (A) water (B) 5% MeOH/water (C) 20% MeOH/water (D) 50% MeOH/water	5 mL 50:50 MeOH-water	5 mL $CHCl_3$
Elution	5 mL EtOAc	5 mL MeOH	5 mL MeOH

[a] mild vacuum for all steps
[b] gravity flow for all steps

3.5. GC–MS/MS technique

A ThermoScientific TRACE 1610 gas chromatograph, equipped with a programmable temperature vaporization injector (PTV), coupled to a TSQ 9610 triple quadrupole mass spectrometer with EI source was used in this study. The instrument was equipped with an Agilent Technologies DB-5MS UI column (Agilent, 30 m x 0.250 mm x 0.25 µm film). Helium served as the carrier gas and nitrogen as the collision gas. A cooler injection temperature was used to avoid the thermal breakdown of the most volatile compounds when exiting the injector. Spectra were recorded and analyzed with Thermo Xcalibur. GC–MS/MS conditions used in this study are outlined in Table 7.

Table 7. GC–MS/MS program used in this study.

Parameter	Programmable Temperature Vaporization
Injection volume	1 µL
Injection temperature	60 °C
Oven temperature	45 °C (2 min. hold) 7 °C/min to 250 °C 10 °C/min to 280°C (2 min. hold)
Total run time	40 minutes
Transfer line temperature	290 °C
Ion source temperature	230 °C

Parameter	Programmable Temperature Vaporization
Ionization method	EI

3.5.1. Optimization of the MRM-method

An explosive mixture solution containing 5 µg/mL of TNT and its degradation products in DCM was used for the method optimization process. After the retention times of the target compounds were obtained in full scan mode (mass range from m/z 40 to 500 and scan time 0.15 s), an MRM method was developed by using the AutoSRM-function. Minimum of three ion transitions were selected for each analyte for optimization. A fixed 0.1-minute retention time window was set for all transitions. The selected transitions are shown in Table 8 and the chromogram of the standard mixture solution in Figure 9.

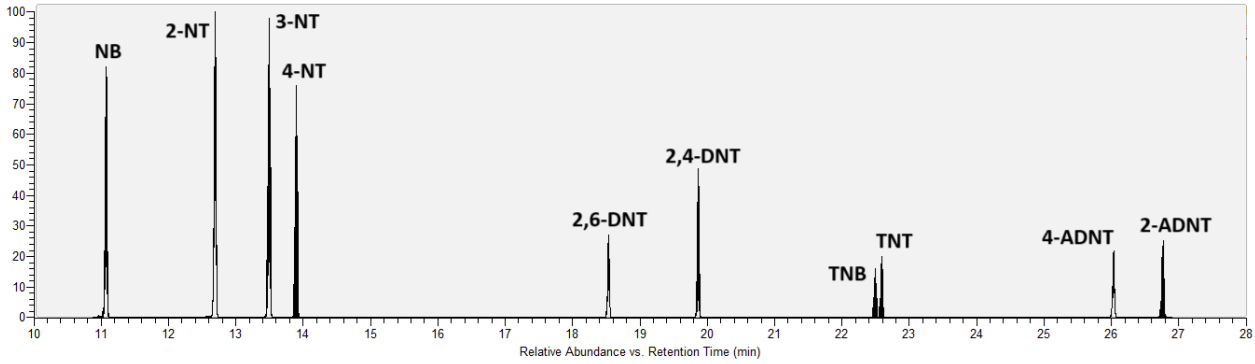


Figure 9. GC–MS/MS MRM chromatogram of the standard solution containing TNT and its degradation products in DCM at a concentration level of 5 µg/mL.

3.6. Quality control

A quality control (QC) sample was measured at the beginning and at the end of each analysis batch to demonstrate appropriate instrument performance and ensure accurate and reliable analytical results. For GC–MS/MS analysis, the recommended quality control sample consist of the following chemicals at concentration level of 5 µg/mL: [69]

- n-alkanes even members, from octane to tetracosane
- trimethylphosphate
- 2,6-dimethylphenol

- 5-chloro-2-methylaniline
- tri-n-butylphosphate
- dibenzothiophene
- malathion
- methyl stereate

3.7. Identification of target compounds

The ion-ratios of the target analytes were calculated by using the MRM data of the standard mixture used for the spiking experiments and the internal standards solutions. HCB was the ISTD used in the extractions following the reference method, while 4-NT-d_7 was used in the extractions with the optimized parameters. Details can be found on Table 8. The HCB transitions (RT = 22.75 min) used were the same as in the Recommended Operating Procedures for CWC-related Analysis, Chapter IX.B. "Analysis of explosives in sediment and soil".[69]

Table 8. MRM conditions for the GC–MS/MS analysis and ion-ratios used to confirm the presence of explosives and internal standards.

Compound	Retention time (min)		Transitions (m/z) and collision energies (eV) in brackets	Ion-ratios
NB	11.07	Q q1 q2	77 → 51 (17) 123 → 77 (15) 123 → 65 (12)	- 73.1% 21.4%
2-NT	12.69	Q q1 q2	120 → 92 (10) 120 → 65 (20) 65.1 → 39.1 (15)	- 78.3% 51.1%
3-NT	13.49	Q q1 q2	91.1 → 65 (13) 137 → 91 (13) 137 → 65 (32)	- 93.4% 26.1%
4-NT d7	13.79	Q q1 q2	144.1 → 114.1 (8) 144.1 → 98.1 (20) 114.1 → 86.1 (8) 114.1 → 86.1 (8)	- 49.3% 47.3%
4-NT	13.92	Q q1 q2	137 → 107 (6) 137 → 91 (19) 137 → 65 (32)	- 52.4% 24.5%
2,6-DNT	18.53	Q q1 q2	165 → 148 (10) 165 → 90 (17) 89.1 → 63 (20)	- 69.0% 50.1%
2,4-DNT	19.86	Q q1 q2	165 → 119 (8) 89 → 63 (17) 165 → 107 (10)	- 47.4% 12.9%

Compound	Retention time (min)		Transitions (m/z) and collision energies (eV) in brackets	Ion-ratios
TNB	22.49	Q	120 → 74 (13)	-
		q1	213 → 120 (28)	23.0%
		q2	213 → 167 (13)	12.7%
TNT	22.58	Q	210 → 164 (6)	-
		q1	210 → 193 (12)	60.0%
		q2	193 → 163 (8)	7.8%
4-ADNT	26.03	Q	197 → 180 (8)	-
		q1	104.1 → 77 (12)	82.5%
		q2	180 → 78 (27)	66.4%
2-ADNT	26.76	Q	197 → 180 (8)	-
		q1	180 → 78 (31)	11.7%
		q2	180 → 163 (6)	0.7%
HCB	22.75	Q	284 → 249 (25)	-
		q1	284 → 214 (35)	76.8%
		q2	284 → 142 (50)	9.4%

Q: quantifier
q: qualifier

With the exception of 4-NT d7 and TNB, the most abundant transition was selected as the quantifier ion and the two other transitions were selected as qualifiers. In the case of 4-NT d7, the most abundant transition 98.1 → 70.1 in DCM, EtOAc-DCM and $CHCl_3$ extracts presented a 'shoulder' in the chromatogram, which could affect the recovery calculations. Therefore, the transition 144.1 → 114.1 was selected as the quantifier transition. For TNB, co-elution with TNT was observed for the transition 120 → 74 in the analyses of the low concentration standard solutions (TNB c = 120 ng/mL) used in the recovery studies, thus the reaction 213 → 120 was selected as the quantifier.

Identification criteria specified by the European Commission Decision (2002/657/EC) were followed in the assessment of the quality of the identifications based on the determined ion-ratios.[72] Tolerances are summarized in Table 9.

Table 9. European Commission Decision criteria for ion-ratios.[72]

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

3.8. Recovery studies

To calculate the relative recoveries (RRs) of all compounds, the single-point internal standard method was used. Unlike external standard methods, this approach accounts for any variances in GC performance. First, standard solutions (n = 3) containing the ISTD (4-NT d7 or HCB), and the relevant analytes were prepared and analyzed with the optimized GC–MS/MS MRM method. These solutions were prepared with the target compounds at concentrations matching the expected levels in the final extracts (i.e. 100% recovery). The concentrations of the analytes were approximately 120 ng/mL for TNT, TNB, ADNTs, and DNTs; 357 ng/mL for NB; and 476 ng/mL for NTs. For the ISTDs, the concentration was 47 ng/mL. The response factor (f_r) was then calculated based on the relationship between the peak area ratio and concentration ratio of the target component (tc) and the ISTD (Equation 1).

$$f_R = \frac{A_{tc}}{A_{ISTD}} \times \frac{C_{ISTD}}{C_{tc}} \quad (1)$$

Following this, analyte concentration in the extract (C_x) was determined according to Equation 2 and relative recovery according to Equation 3:

$$C_x = \frac{A_x}{A_{ISTD}} \times \frac{C_{ISTD}}{f_R} \quad (2)$$

$$RR\ \% = \frac{C_x}{C_{tc}} \times 100 \quad (3)$$

3.9. Results and Discussion

3.9.1. Identification of target compounds

As previously described, selected ion ratios were used to confirm identification of analytes in the extracts. Figure 10 illustrates how verification of the presence of 4-NT in a spiked sediment sample extracted with DCM was conducted. The retention time difference between reference and sample is within the allowed limit of ± 0.1 min. The ion ratios of the two qualifier transitions (137 → 91 and 137 → 65) and the quantifier transition (137 → 107) were 51.4% and 24.6%, respectively and the allowed limits for these ratios were 52.4% ± 10.5 and 24.5% ± 6.1%, respectively. Accordingly, identification was accepted.

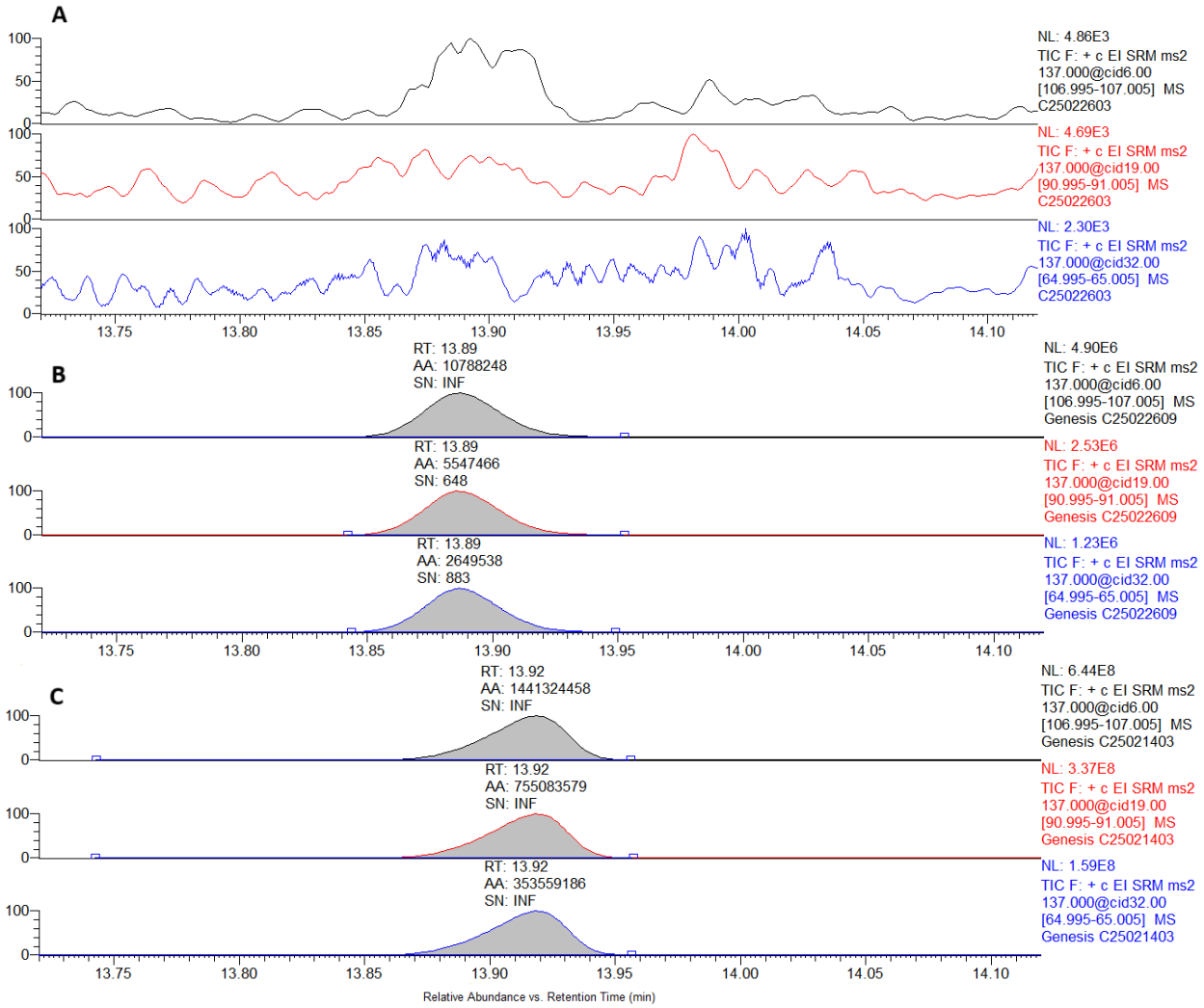


Figure 10. Example of identification criteria for 4-NT using GC–MS/MS. The presented MRM chromatograms are: (A) blank sediment sample, (B) simulated contaminated sediment sample, (C) standard explosive mixture solution containing 4-NT at 20 µg/mL.

Ideally, the two ion ratios should fall within the allowed limits to meet the identification criteria, as in the example shown. However, due to the complex sample matrix and low analyte concentrations (ppb), having one ion ratio within the range was considered sufficient to confirm identification.

None of the extracted blank samples contained any of the ten target analytes, and the ISTD was correctly identified in all cases. Therefore, it was concluded that the studied marine sediment contained no detectable limits of TNT and its derivatives.

Most compounds were successfully identified in the spiked sediment extracts with the exception of TNB and NB. TNB was not detected in the DCM extracts analyzed according

to the ROP and NB was not detected in the DCM extracts prepared using the optimized parameters. As for TNB, no peaks were detected, possibly due to low extraction efficiency leading to very low concentrations. For NB, peaks were detected for all of the three transitions, but both ion-ratios were out of tolerance, meaning that the identification was rejected. This rejection may be linked to background interference.

Table 10. Compounds identified in the spiked sediment extracts.

Compounds	DCM ROP	DCM opt	EtOAc	EtOAc-DCM	$CHCl_3$
NB	✓	-	✓	✓	✓
2-NT	✓	✓	✓	✓	✓
3-NT	✓	✓	✓	✓	✓
4-NT	✓	✓	✓	✓	✓
2,6-DNT	✓	✓	✓	✓	✓
2,4-DNT	✓	✓	✓	✓	✓
TNB	-	✓	✓	✓	✓
TNT	✓	✓	✓	✓	✓
4-ADNT	✓	✓	✓	✓	✓
2-ADNT	✓	✓	✓	✓	✓
HCB	✓	NA	NA	NA	NA
4-NT d7	NA	✓	✓	✓	✓

NA = not applicable

3.9.2. Quantitation of analytes

After identification, compounds were quantified. Table 11 shows the quantitation results for the compounds present in extracts from spiked sediment samples.

TNB was identified in the $CHCl_3$ extracts as shown in Table 10; however, quantitation was not possible because peaks for the selective transitions were not observed in the low-concentration standard solutions (c = 120 ng/mL) used for quantitation. Since this concentration is generally not too low for GC based analysis, and other analytes were successfully identified in the same solution, the absence of TNB peaks may be due to systematics. This assumption could be verified by analyzing another solution prepared using the same method.

As previously discussed, NB and TNB were not identified in the DCM opt and DCM ROP extracts, respectively; consequently, these compounds were not quantified.

Table 11. Mean analyte concentration ± standard deviation (n = 3) in ng/mL in spiked sediment extracts.

Compounds	DCM ROP	DCM opt	EtOAc	EtOAc-DCM	$CHCl_3$
NB	104.9 ± 4.0	NQ	161.7 ± 7.9	144.4 ± 12.2	125.4 ± 7.7
2-NT	142.0 ± 8.8	150.0 ± 9.8	261.6 ± 11.1	223.8 ± 15.1	209.3 ± 10.9
3-NT	147.2 ± 10.4	138.5 ± 9.0	294.9 ± 13.2	244.2 ± 12.3	231.2 ± 12.2
4-NT	100.4 ± 7.1	184.5 ± 10.7	257.0 ± 12.1	234.1 ± 12.6	215.8 ± 6.5
2,6-DNT	42.0 ± 3.2	49.2 ± 3.2	63.5 ± 2.8	62.8 ± 0.5	67.6 ± 1.2
2,4-DNT	42.0 ± 2.1	48.1 ± 2.4	63.7 ± 2.1	67.1 ± 1.6	71.8 ± 4.9
TNB	NQ	27.1 ± 5.2	54.4 ± 1.2	55.4 ± 3.0	NQ
TNT	46.0 ± 2.8	41.1 ± 5.9	60.9 ± 2.3	63.2 ± 3.6	75.5 ± 7.1
4-ADNT	39.9 ± 2.7	39.8 ± 3.8	16.5 ± 4.3	42.9 ± 9.5	13.9 ± 3.6
2-ADNT	78.3 ± 4.0	108.0 ± 7.9	74.8 ± 6.0	106.1 ± 8.1	103.7 ± 13.2

NQ = not quantified

The Recommend Operating Procedure for analysis of explosives in sediment and soil from the Blue Book states that the method enables detection of the target compounds at concentration levels of 1 ng/mL.[69] As the GC–MS/MS method developed in this study was not validated, the limits of detection (LODs) and limits of quantification (LOQs) for the analytes remain unknown, making it not possible to confirm whether the reported LOD was achieved for the target analytes in all evaluated procedures.

3.9.3. Extraction

3.9.3.1. Reference method

Nine compounds were successfully recovered in the sediment extraction following the ROP, with TNB being the only analyte not detected in the extracts. Although TNT recovery (38.4% ± 2.3%) was higher than the one obtained in the DCM extraction with the tested parameters (34.3% ± 4.9%), this reference method demonstrated inferior performance when all recoveries were considered (Figure 11). A more detailed assessment of the evaluated parameters is presented in the next section.

3.9.3.2. Optimized method

A comparison between the relative recoveries obtained from the studied compounds by extraction with DCM following the ROP and with the proposed optimizations (centrifugation conditions, shaking speed, filter pore size, drying salt, extract overnight storage and internal standard) is presented in Figure 11. As already mentioned, both ion ratios for NB exceeded the allowed limits, explaining why there is no recovery associated to this compound in the optimized extraction.

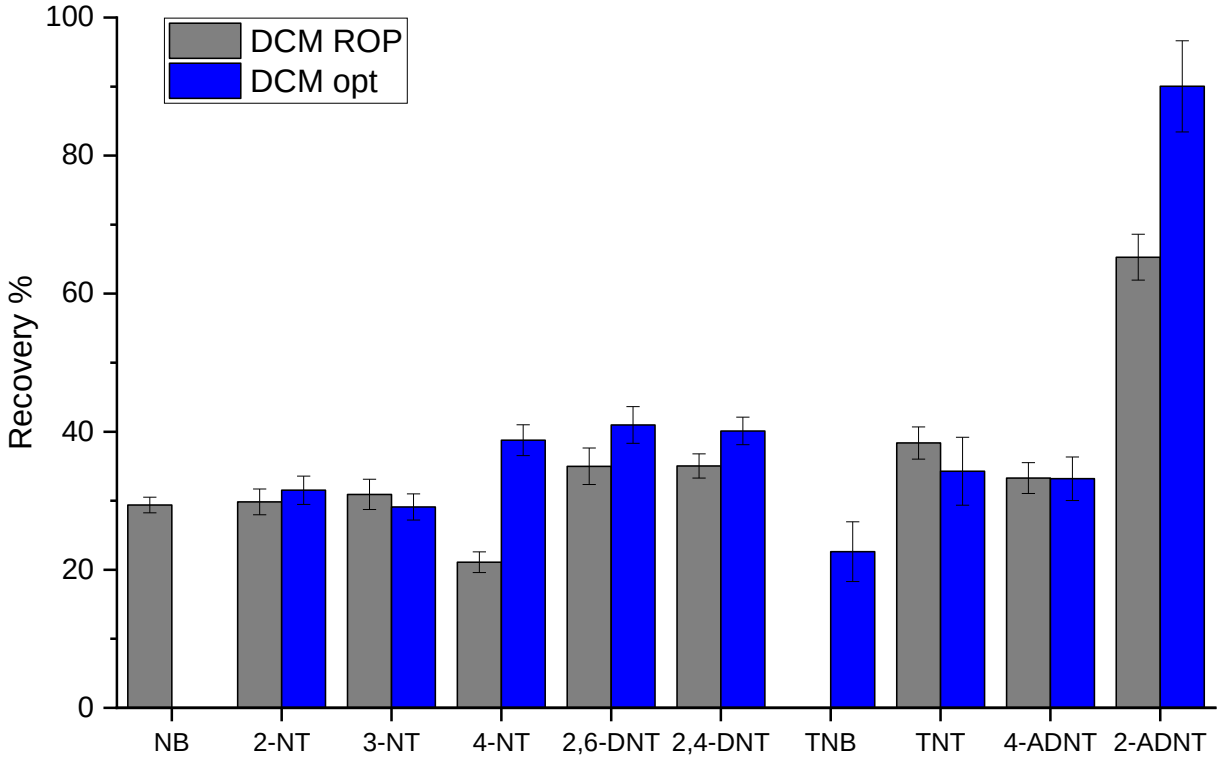


Figure 11. Mean analyte recoveries ± standard deviation (n = 3) from spiked sediment samples extracted with dichloromethane according to the Recommend Operating Procedure from the Blue Book (DCM ROP) and following the suggested optimizations (DCM opt).

To fully determine the most effective extraction method for the studied compounds, each parameter would have to be individually assessed. This would require a robust study with multiple extractions following the same protocol, with modifications made to only one variable at a time. Due to time constraints, such detailed study was not conducted.

3.9.3.2.1. Centrifugation

After centrifugation according to the established procedure, the pore water appeared turbid, implying that extremely small particles did not separate due to insufficient centrifugal force. Additionally, water was not completely removed from the sample and consequently the estimated pore water content was lower than that calculated when the optimized conditions were used (Table 12). Excess water can adversely affect the extraction performance of the organic solvent. Therefore, centrifugation for 5 minutes at 3000 x g is advised.

Table 12. Pore water content estimation in the analyzed samples.

Method	Centrifugation conditions	Pore water content
DCM ROP	1000 x g, 3 minutes	19.3 %
DCM opt	3000 x g, 5 minutes	28.9%

3.9.3.2.2. Shaking

With respect to shaking, high speed is recommended, as it favors the breakdown of solid agglomerates and ensures that more surface area of the solid is exposed to the solvent, resulting in better extraction efficiency.[73] This could be one of the explanations for the increment in extraction yield observed for 2-NT, 4-NT, 2,6-DNT, 2,4-DNT and 2-ADNT when the shaking speed was 2000 rpm instead of 1000 rpm.

3.9.3.2.3. Filter pore size

Altering the filter pore size from 0.45 μm to 0.2 μm does not result in an immediate positive outcome (e.g. higher extraction efficiency), but rather extends the column lifetime, as this change can reduce clogging and contamination issues caused by small particles present in the matrix. A smaller pore-sized filter also decreases the risk of the syringe getting clogged during the injection into the analytical instrument.

3.9.3.2.4. Drying

Magnesium sulfate and sodium sulfate were both effective in removing residual water from the organic extracts. As for the drying conditions, the extracts were allowed to sit

overnight in the refrigerator or at room temperature. Although the results do not indicate any preservation issues for extracts stored at ambient temperature in the short-term, future experiments should ensure that samples are refrigerated at all times to maintain their integritity, prevent contamination, and avoid degradation of heat-sensitive compounds.

3.9.3.2.5. Internal standard

4-NT d7 and HCB performed well in all experiments. Isotopically labeled ISTD is preferred due to its very similar chemical structure and physicochemical properties to the target analytes. The only issue observed with 4-NT d7 was a shoulder on the peak corresponding to the transition 98.1 → 70.1 in DCM, EtOAc-DCM, and $CHCl_3$ extracts, suggesting co-elution with a structurally related compound or matrix interference. However, this can be solved by selecting an alternative abundant transition.

HCB is not chemically similar to any of the compounds investigated but, in general, it works well as an ISTD for this analysis, since it is chemically and thermally stable, it is not present in the sample and can be more easily obtained than 4-NT d7. Additionally, HCB is widely used in environmental sample analyses. It is also possible to use several internal standards in a sample, especially if target analytes have varying properties and retention times. This was not, however, deemed necessary in this study.

3.9.3.2.6. Solvent

Figure 12 shows the recovery results for extractions carried out with DCM, EtOAc, 50:50 EtOAc-DCM and $CHCl_3$ and the optimized parameters. Among the four solvents tested, the one that most effectively extracted the targeted compounds from spiked sediment samples was the EtOAc-DCM mixture, showing the highest relative recovery for six compounds (NB, 2-NT, 3-NT, 4-NT, TNB and 4-ADNT), an average recovery of 51.9%, and an average standard deviation of 3.4%. These results suggest that the combination of solvents, with differing polarity and lipophilicity, can be advantageous in SLE. Extraction with $CHCl_3$, EtOAc, and DCM resulted in average recoveries ± average standard deviations of 45.0% ± 3.3%, 49.4% ± 2.6%, and 36.1% ± 3.0%, respectively.

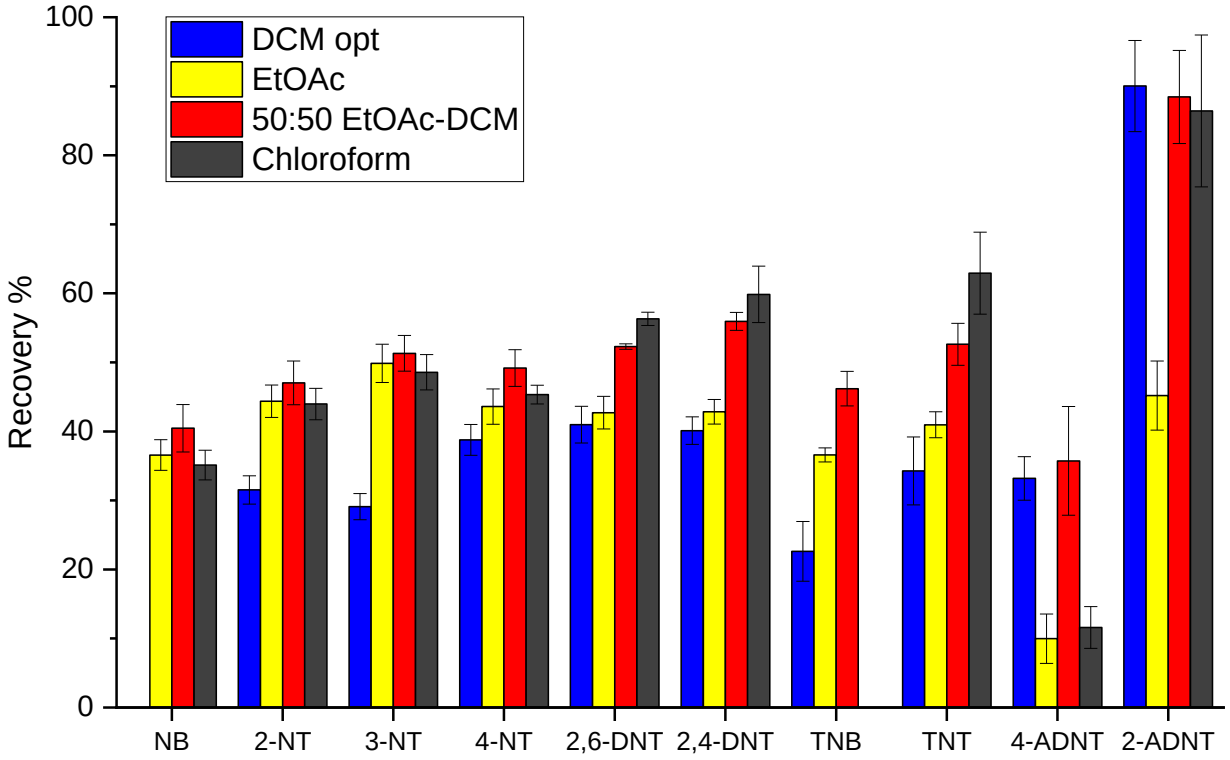


Figure 12. Mean analyte recoveries ± standard deviation (n = 3) from spiked sediment samples extracted with DCM, EtOAc, 50:50 EtOAc-DCM and $CHCl_3$ using the optimized parameters detailed in Table 4.

In the case of DCM and $CHCl_3$ extractions, the organic layers were under the sediment (bottom phase), making it difficult to access the extract. In the extractions with EtOAc-DCM, and EtOAc, the extract was on the upper phase, thereby facilitating the collection of the organic layer and preventing sediment loss in the glass pipette.

DCM and EtOAc-DCM extracts were rapidly concentrated compared to $CHCl_3$ and EtOAc due to the considerably lower boiling point of DCM. On some occasions, evaporation was unintentionally carried out to dryness, which may have contributed to the low recoveries observed for certain analytes, particularly the more volatile ones. Analyte loss can happen even if this step is carefully executed, as demonstrated by Thomas et al. (2018). In that study, low TNT recoveries observed for positive controls extracted by syringe filtration were attributed to, among other factors, losses occuring during the evaporation step.[19]

For TNT, the primary compound studied, $CHCl_3$ extraction yielded the highest recovery corresponding to 62.9%, while DCM extraction resulted in the lowest one, at 34.3%.

Before comparing the results with literature data, it is important to recognize that the methods used in previous studies differ from the ones implemented in this thesis. Many parameters can influence recoveries, including extraction conditions – particularly the solvent used – and the spiking level used in each experiment, as matrix effects tend to be more pronounced at lower target compound concentrations. Gordon et al. (2018) spiked sediment samples with TNT to a concentration of 1 mg/g while Dawidziuk et al. (2018) spiked their samples at a slightly higher concentration of 1.25 mg/g.[12,16] SLEs were performed with $CHCl_3$ and the recoveries obtained were 90.5% and 77.5%, respectively. However, in the present study, TNT concentration in sediment was 25 ng/g which is 40,000 times lower than 1 mg/g. Thereby, this decrease in recovery could be primarily attributed to the low spiking level.

Dawidziuk et al. (2018) have also reported recoveries for 2,4-DNT (83.5%) and 2,6-DNT (87.4%), and both quantities were higher than the ones obtained in this work: 59.9% and 56.3%, respectively. Multiple factors could explain the variability, including differences in the GC–MS/MS parameters and the absence of an evaporation step in their study.

While key properties, such as polarity and solubility, helps in estimating the potential extraction outcomes for targeted compounds, experimental results are not always straightforward. Additional interactions may occur that cannot be fully understood based solely on initial tests. One example is the high discrepancy in recovery rates between the isomers 4-ADNT and 2-ADNT obtained in this study, which could be attributed not only to their different lipophilicity (log Kow 2-ADNT = 1.94; log Kow 4-ADNT= 1.79),[67,68] but also interactions with matrix components or varying time required to partition into the solvent.

An efficient and practical extraction method should be able to extract all target compounds, even those at low concentrations, provide good recoveries with low standard deviations and use solvents with low boiling points and minimal toxicity whenever possible. Therefore, SLE with 50:50 EtOAc-DCM and the optimized parameters was considered the best method for detection and quantification of TNT and its degradation compounds in marine sediment samples.

3.9.4. Clean-up

All SPE experiments with Oasis HLB and CHROMABOND Easy using EtOAc extracts failed. Analytes possibly passed directly through the cartridge after sample loading due to the high EtOAc polarity and thus were not retained to the stationary phase. As for the second experiment that included solvent exchange from EtOAc to diluted acetone, analytes were not detected either, in this case possibly due to the low concentration and/or loss of analytes during the analytical process (e.g. evaporation). With Si-1, explosives were not detected either, or the experiment may have failed for the same reasons as those mentioned above.

3.10. Limitations and future prospects

Despite the promising results, this study presented certain limitations, particularly the time available for the experimental research. This constraint restricted the extent of method and sample preparation optimization, the number of replicates performed in each test, and limited the degree of method validation. Moreover, certain challenges encountered during the course of this work, such as unexpected missing transitions of certain compounds, could not be investigated in detail.

Future research should prioritize the systematic evaluation of extraction parameters – one at a time – to maximize the analytical outcomes (e.g., higher recoveries, lower LODs) and, consequently, enhance the reliability, accuracy, and sensitivity of methods used to detect and quantify TNT and its degradation products in sea-dumping sites. In addition, sample clean-up procedures should be first evaluated with clean solvents and reference standards to ensure efficient use of time and analytical resources. Lastly, real-world environmental samples from known contaminated areas should be analyzed by the developed/improved method to assess its performance under realistic matrix conditions.

3.11. Conclusion

A reference GC–MS/MS method for the determination of TNT and nine degradation compounds in marine sediment samples was successfully improved but not validated due to time constraints.

No target compounds were detected in blank sediment extracts obtained by any of the evaluated extraction procedures, implying that the analytes were either absent or in non-detectable low concentrations in the sediment sample studied.

With the reference extraction method (DCM ROP), TNB was not detected in the spiked sediment extracts, but the ISTD was correctly identified, meaning that this absence was not related to instrumental issues. With the optimized parameters, NB was not identified because the transitions did not meet the identification criteria. Excluding these two compounds, analytes were more efficiently extracted via the DCM optimized extraction, with an average recovery of 42.2%, than following the Recommended Operating Procedure where the average recovery was 36.1%.

With respect to the sample preparation optimization, those variables that possibly contributed to enhance extraction efficiency or improve analytical performance were centrifugation for 5 minutes at 3000 x g, 2000 rpm shaking speed and filtration with 0.2 µm pore size filter. Since all parameters under optimization were evaluated concurrently, the influence of extract drying conditions on the integrity and stability of the investigated analytes was not determined. As for the drying salts and ISTDs investigated, the tested compounds proved to be suitable, and no clear preference can be established between them with the current results.

Among the extraction solvents investigated in combination with the optimized parameters, extractions carried out with 50:50 EtOAc-DCM provided the highest recovery results for most compounds. The relative recoveries ranged from 35.7% (4-ADNT) to 88.4% (2-ADNT), whereas in the EtOAc extraction, the obtained recoveries were between 13.7% (4-ADNT) and 62.3% (2-ADNT). Analytes extracted using these two solvents were successfully detected and quantified. In contrast, TNB was the only analyte not quantified in the $CHCl_3$ extracts, as its peaks were not observed in the low concentration standard solution used for quantitation. However, the concentrations of 2,6-DNT, 2,4-DNT, and TNT in the $CHCl_3$ extracts were higher than those obtained in the other evaluated extractions (67.6 ng/mL, 71.8 ng/mL and 75.5 ng/mL, respectively).

After SLE optimization, Oasis HLB, CHROMABOND Easy, and Strata Si-1 were evaluated as part of the development of a clean-up procedure for the spiked sediment extracts. All

experimental attempts were unsuccesuful. Analytes were not detected in any of the extracts, probably due to evaporations losses, or, as in the first experiments perfomed with EtOAc extracts, because sediment extracts were in a high elution strenght solvent.

While this work provides only preliminary results concerning sample preparation and does not evaluate validation parameters, it offers valuable insights that can guide further experiments for the detection and quantitation of TNT and its breakdown products in marine sediment samples from dumping sites using GC–MS/MS.

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