pan-European Management of Biological toxin incidents through standaRdisAtion initiatives for Crisis response Enhancement

D3.3

Analysis of Target Biotoxins and Analysis ROPs - 1st Iteration

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Abstract	This deliverable presents the first iteration of an analytical framework for the detection and identification of selected biotoxins developed in EMBRACE Task 3.2. The work focuses on laboratory-based qualitative identification and defines a harmonised analytical concept combining LC–MS-based workflows for low-molecular-weight toxins with tiered immunological, functional, and proteomic approaches for protein toxins. The framework is implemented through draft analytical workflows representing current method development status rather than fully validated procedures. The deliverable establishes an orthogonality-based identification concept, outlines analytical readiness across the toxin panel, and provides the basis for future development of Recommended Operating Procedures (ROPs) in subsequent project phases.
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V1.0	31.03.2026	VER	Final version submitted

Abbreviations

Adda / ADDA	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid
BoNT	botulinum neurotoxin
BWC	Biological Weapons Convention
CBRNE	chemical, biological, radiological, nuclear, and explosives
CCα	decision threshold (critical level)
CCβ	detection capability
CWC	Chemical Weapons Convention
DoA	Description of Action
DON	deoxynivalenol
ELISA	enzyme-linked immunosorbent assay
Endopep	endopeptidase-based functional assay
EQuATox	European Quality Assurance for Detection of Biological Toxins
ESI	electrospray ionisation
EuroBioTox	European biotoxin project / proficiency-testing initiative cited in the deliverable
GC–MS	gas chromatography–mass spectrometry
HILIC	hydrophilic interaction liquid chromatography
HMW	high molecular weight
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectrometry
ISO/IEC	International Organization for Standardization / International Electrotechnical Commission
IUPAC	International Union of Pure and Applied Chemistry

LC	liquid chromatography
LC–MS/MS	liquid chromatography–tandem mass spectrometry
LLE	liquid–liquid extraction
LMW	low molecular weight
LOQ	limit of quantification
MALDI-TOF	matrix-assisted laser desorption/ionisation time-of-flight
MBA	mouse bioassay
MC-LR	microcystin-LR
MDAs	monoester diterpenoid alkaloids
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NOD	nodularin
OPCW	Organisation for the Prohibition of Chemical Weapons
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PPIA	protein phosphatase inhibition assay
PRM	parallel reaction monitoring
PSTs	paralytic shellfish toxins
QA/QC	quality assurance / quality control
QqQ	triple-quadrupole mass spectrometer
QTOF	quadrupole time-of-flight
RCA120	Ricinus communis agglutinin 120 kDa

RIP	ribosome-inactivating protein
RIP-II	type II ribosome-inactivating protein
ROP	Recommended Operating Procedure
RP-LC	reversed-phase liquid chromatography
SAB	Scientific Advisory Board
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SPE	solid-phase extraction
SRM	selected reaction monitoring
STX	saxitoxin
T-2	T-2 toxin
UHPLC	ultra-high-performance liquid chromatography
UNODA	United Nations Office for Disarmament Affairs
UNSGM	United Nations Secretary-General's Mechanism
UPLC	ultra-performance liquid chromatography
WHO	World Health Organization

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EXECUTIVE SUMMARY

Purpose.
This deliverable (D3.3) documents the first iteration of EMBRACE Task 3.2 analytical work on target biotoxins and establishes the design principles for future analysis ROPs. The focus is on laboratory-based verification and qualitative identification, not field sampling. The work adopts an orthogonality-first analytical philosophy and defines reporting principles aligned with international expectations for defensible and interpretable identification.

Scope of this iteration.

The targets defined in the Description of Action are treated in this deliverable as canonical exemplars of broader toxin classes, forming the basis for structured method development in the first iteration and later scope expansion.

- **Low-molecular-weight (LMW) toxins:** A harmonised LC–MS-centred workflow has been established, combining high-resolution screening with targeted LC–MS/MS follow-up. Orthogonality is achieved through complementary chromatographic modes (HILIC and RP) and, where applicable, alternative analytical routes (e.g. derivatisation or oxidation-based methods for PSTs).
- **Protein toxins:** A tiered analytical strategy has been defined, combining immunological screening, selective enrichment, functional assays, and peptide-level LC–MS/MS confirmation. Development is ongoing, with key analytical modules already defined but not yet fully consolidated across matrices and scope.
- **Reporting:** A qualitative reporting framework has been established, including controlled result categories, minimum reporting content, QA/QC requirements, and explicit statement of limitations, compatible with OPCW and UNSGM-oriented expectations for laboratory outputs.

Key outcomes. The first iteration has established:

- a unified analytical workflow with a common intake concept and two harmonised analytical branches (for LMW and protein toxins);
- a consistent evidential framework based on orthogonality and qualitative decision rules;
- a structured QA/QC and reporting approach for defensible qualitative identification; and
- a readiness-based prioritisation of further analytical development across the toxin panel.

Limitations and current readiness. Analytical readiness is heterogeneous across targets and remains scope-dependent and matrix-dependent. Confirmatory capability has been established for selected targets within defined scope, while others remain at screening-ready or confirmation-under-development stage.

Attribution-oriented analysis has been considered at the level of analytical prerequisites, but no attribution-ready workflows are yet available. Progress in this area is expected to be constrained by the availability of suitable comparative materials and datasets.

Reference materials, interlaboratory validation, and matrix-specific performance data remain uneven across the panel. These limitations are addressed in the current iteration through transparent scope definition and explicit reporting of uncertainty and analytical constraints.

1 INTRODUCTION

1.1 Background and context

Biotoxins comprise a chemically and structurally diverse group of naturally occurring toxic substances produced by plants, bacteria, fungi, and marine organisms. Several of these toxins are of particular concern in the context of chemical and biological security because of their high toxicity, accessibility from natural sources, and potential for deliberate misuse (Dorner et al., 2016; OPCW, 2023). Examples include plant-derived protein toxins such as ricin, bacterial neurotoxins such as botulinum neurotoxins, marine neurotoxins such as saxitoxin and related paralytic shellfish toxins (PSTs), cyanobacterial toxins such as microcystins, and a range of toxic natural products including trichothecene mycotoxins and plant alkaloids such as aconitine and strychnine (Bouaïcha et al., 2019; Gawankar et al., 2024).

The identification of toxic agents in environmental, food, or biomedical samples is a critical capability in the investigation of suspected poisoning incidents, deliberate toxin release events, or other public health emergencies. In such contexts, analytical laboratories must be able to detect and identify a wide range of toxic compounds in complex matrices while meeting stringent requirements for analytical reliability and evidential confidence (OPCW, 2023; Rasetti-Escargueil et al., 2024). Forensic identification of toxins therefore requires analytical workflows capable of combining high sensitivity with robust confirmation criteria.

International verification frameworks have recognised the analytical challenges associated with toxin identification. For example, several naturally occurring toxins, including ricin and saxitoxin, are specifically listed under Schedule 1 of the Chemical Weapons Convention (CWC) due to their extreme toxicity and potential for misuse (OPCW, 2013). Analytical laboratories supporting chemical weapons investigations or international verification mechanisms therefore require validated analytical methods capable of identifying such toxins with high confidence, typically supported by orthogonal analytical evidence (OPCW, 2023).

The analytical identification of biotoxins presents several technical challenges. First, toxins vary widely in molecular size and chemical properties, ranging from small organic molecules to complex cyclic peptides and large protein toxins. As a consequence, no single analytical technique can address all toxin classes. Instead, laboratories typically rely on a combination of analytical approaches including liquid chromatography–mass spectrometry (LC-MS), immunological detection methods, and functional assays that measure toxin activity. The use of orthogonal analytical techniques is widely regarded as essential for achieving reliable identification and reducing the risk of false positives in forensic or verification contexts (Dorner et al., 2016; OPCW, 2023).

Within the EMBRACE project, strengthening analytical capabilities for the detection and identification of biotoxins represents an important component of improving preparedness for chemical and biological threat scenarios. Work Package 3 (WP3) focuses on the analytical detection and forensic investigation of biotoxins, including the development of analytical workflows suitable for incident-related samples such as environmental swabs, powders, food extracts, and biological fluids. In particular, Task 3.2 aims to establish procedures and methodological guidelines for forensic biotoxin

analysis, building on existing best practices and adapting them to the toxin panel addressed in the project (OPCW, 2023).

The present deliverable contributes to this objective by providing a structured overview of the analytical strategies and methodological frameworks currently under development within WP3. The work described here builds upon established analytical approaches, while also documenting emerging workflows that will serve as the basis for future Recommended Operating Procedures (ROPs) for forensic biotoxin analysis within the EMBRACE framework.

1.2 Objectives of the deliverable

The objective of **Deliverable D3.3** is to provide a structured overview of analytical strategies and methodological frameworks for the detection and confirmation of selected biotoxins within the EMBRACE project. The deliverable represents an intermediate step in the development of harmonised analytical procedures for forensic biotoxin analysis within **Work Package 3 (WP3)**.

More specifically, the deliverable aims to:

1. **Define the scope of biotoxins addressed within the first iteration of WP3 analytical work**, including both protein toxins and low-molecular-weight toxins relevant to the EMBRACE threat landscape.
2. **Review and evaluate existing analytical approaches** applicable to the detection and confirmation of these toxins. This includes established analytical techniques such as liquid chromatography–mass spectrometry (LC-MS/MS), high-resolution mass spectrometry (HRMS), immunological detection methods (e.g., ELISA), and functional assays that measure toxin activity.
3. **Assess the suitability of these analytical approaches for forensic and incident-related sample matrices**, including environmental samples, powders, food extracts, and biological fluids.
4. **Identify analytical gaps and methodological challenges** that must be addressed before harmonised analytical procedures can be implemented across participating laboratories.
5. **Document analytical workflows currently under development within the project**, including both operational method frameworks and emerging analytical concepts. These workflows describe practical strategies for toxin detection and confirmation and are included in the annexes in a protocol-style format.
6. **Establish a preliminary readiness assessment of analytical methods** for the toxin panel addressed in the first phase of WP3 work.

An important objective of this deliverable is therefore to **provide the conceptual and technical foundation for the development of Recommended Operating Procedures (ROPs)** for forensic biotoxin analysis in later stages of the project. While several analytical workflows have already been drafted in a format resembling laboratory protocols, they should currently be regarded as development frameworks rather than finalised ROPs. Further experimental validation, optimisation, and interlaboratory testing will be required before such procedures can be formalised.

In this sense, the present deliverable serves two complementary purposes: it documents the current state of analytical method development within WP3, and it establishes a methodological roadmap for the continued development of harmonised forensic biotoxin analysis procedures within EMBRACE.

1.3 Relation to the Description of Action

This deliverable contributes directly to the objectives of Task 3.2 – "**Procedures and guidelines for forensic analysis of biotoxins**" within Work Package 3 (WP3) of the EMBRACE project.

According to the Description of Action (DoA), Task 3.2 aims to develop analytical procedures and methodological guidelines for the forensic investigation of biotoxins. The task includes the evaluation and improvement of existing analytical methods, the development of new analytical workflows where necessary, and the establishment of harmonised approaches for the detection and confirmation of biotoxins in incident-related samples.

The DoA specifies that the analytical work within Task 3.2 will address several key components of forensic biotoxin analysis, including:

- sample preparation strategies for relevant matrices defined in Task 3.1, such as environmental samples, powders, food extracts, and biological fluids,
- analytical detection methods, including liquid chromatography–mass spectrometry (LC-MS/MS), immunological assays such as ELISA, and complementary analytical techniques,
- analytical confirmation criteria compatible with existing verification frameworks,
- and chemical forensic approaches, including the potential use of impurity profiling to support attribution analysis.

Deliverable D3.3 represents an intermediate milestone in the implementation of these objectives. The deliverable documents the current state of analytical method development within WP3 and provides a structured assessment of analytical approaches relevant to the toxin panel addressed in the EMBRACE project. In particular, it:

- summarises analytical strategies currently being developed for both protein toxins and low-molecular-weight toxins,
- reviews applicable analytical technologies and workflows,
- identifies methodological gaps and constraints, and
- evaluates the readiness of candidate analytical approaches for future procedural development.

The analytical workflows described in the annexes of this deliverable represent early protocol-style implementations of these strategies. While these workflows already reflect practical analytical approaches, they are not yet formalised as Recommended Operating Procedures (ROPs). Instead, they serve as method development frameworks that will inform the drafting of harmonised ROPs during the later stages of Task 3.2.

In this way, Deliverable D3.3 provides the analytical and methodological basis for the continued development of forensic biotoxin analysis procedures within EMBRACE, ensuring that future ROPs will be grounded in a systematic evaluation of analytical capabilities and constraint.

2 SCOPE AND METHODOLOGICAL FRAMEWORK

2.1 Definition of target biotoxins in EMBRACE

Biological toxins are poisonous chemical substances produced by living organisms such as plants, animals, bacteria, fungi, and algae. In contrast to many synthetic toxic chemicals, toxins are often categorised according to the organism that produces them rather than strictly by their chemical structure. From an analytical perspective, however, toxins encompass an extremely wide structural range, extending from small low-molecular-weight molecules to peptides and large protein toxins.

Biological toxins exert their toxic effects through interactions with biological macromolecules such as receptors, enzymes, and nucleic acids. These interactions interfere with essential cellular processes including signal transduction, protein synthesis, and metabolic regulation. The resulting toxic effects vary widely depending on the toxin class and mechanism of action, ranging from moderate toxicity to extremely potent biological activity (Dorner et al., 2016). Among the most potent known toxins are botulinum neurotoxins (Gill, 1982), which act as zinc-dependent proteases that disrupt neurotransmitter release (Arnon et al., 2001).

Because of their high toxicity and potential for misuse, several biological toxins are addressed within international arms-control frameworks. For example, ricin and saxitoxin are listed under Schedule 1 of the Chemical Weapons Convention (CWC) due to their extreme toxicity and potential for use as chemical weapons agents (OPCW, 2013). At the same time, toxins such as botulinum neurotoxins are also considered within the scope of the Biological Weapons Convention (BWC) because they are produced by microorganisms and can be associated with biological warfare contexts (Arnon et al., 2001). Biological toxins therefore occupy an important conceptual space at the interface between classical chemical and biological threat agents.

Within the EMBRACE project, analytical work in Work Package 3 focuses on a defined set of representative biotoxins covering different toxin classes and analytical challenges. These targets include both high-molecular-weight (HMW) protein toxins and low-molecular-weight (LMW) toxins, reflecting the need to develop analytical workflows that remain effective across widely differing molecular structures and physicochemical properties.

An introduction to the individual toxins addressed in this work is provided in **Section 3.2** and **Annex A.**

2.1.1 Criteria for inclusion

The biotoxins addressed in EMBRACE were selected through a risk-informed assessment designed to ensure scientific relevance, operational feasibility, and alignment with international chemical security and non-proliferation priorities.

The final target panel represents a balance between several key considerations:

- Credible threat potential, including toxins historically associated with chemical or biological security concerns.
- Relevance to environmental, food, and public-health scenarios, including toxins that may realistically occur in natural contamination events or deliberate poisoning incidents.

- Analytical feasibility, ensuring that robust analytical detection and confirmation methods can be developed for realistic matrices.
- Regulatory and policy significance, including alignment with international arms-control discussions and scientific advisory activities.

A subset of the EMBRACE targets corresponds to toxins highlighted in activities of the OPCW Scientific Advisory Board (SAB) on biotoxin verification capabilities (OPCW, 2023). In particular, ricin and saxitoxin are frequently used as representative examples of high-molecular-weight and low-molecular-weight toxins in analytical verification discussions. Their inclusion in the EMBRACE toxin panel therefore supports compatibility with longer-term international verification priorities.

Additional toxins were selected to broaden the applicability of the analytical framework to civil-protection, forensic, and public-health contexts. These include toxins relevant to environmental exposure scenarios, such as cyanobacterial toxins and marine neurotoxins, which are discussed in public-health guidance from organisations such as the World Health Organization (WHO).

An important design principle was to ensure that the selected toxin panel spans multiple toxicological mechanisms and molecular classes. This approach avoids optimisation of analytical methods for a single toxin archetype and instead supports the development of analytical workflows that remain applicable across a wide spectrum of toxin chemistries. The resulting panel therefore includes toxins that differ substantially in molecular size, structural complexity, and biological mode of action, ranging from small guanidinium alkaloids such as saxitoxin to large protein toxins such as ricin.

Finally, toxin selection was constrained by practical considerations related to analytical method development. All targets were required to have available reference materials, certified standards, or suitable surrogates to enable credible method development and performance characterisation. Because **Work Package 3** currently represents the only analytical laboratory within EMBRACE, the selected toxins also needed to allow analytical confirmation workflows that can be implemented within a single-laboratory framework, while still producing results compatible with internationally recognised expectations for unambiguous identification.

These selection principles directly informed the **analytical strategies described in Section 2.2.**

2.1.2 Relationship to regulatory and operational frameworks (CBRN, public health, forensic)

The toxin panel and analytical framework described in this deliverable are designed to remain compatible with several international operational and regulatory contexts relevant to toxin identification.

CBRN and arms-control context

Biotoxins occupy a unique position within international arms-control regimes because they lie at the interface between chemical and biological agents. Within the Chemical Weapons Convention, certain toxins such as ricin and saxitoxin are explicitly listed under Schedule 1 (OPCW, 2013). Analytical approaches used in EMBRACE therefore consider the general verification principles discussed in the

context of OPCW Scientific Advisory Board activities, including the importance of unambiguous identification and the use of orthogonal analytical evidence (OPCW, 2023).

Public-health and environmental relevance

Several toxins included in the EMBRACE panel are also relevant in environmental and food-safety contexts. Cyanobacterial toxins and marine toxins, for example, are well-known causes of natural poisoning events associated with harmful algal blooms and contaminated seafood. Public-health guidance from organisations such as the **World Health Organization (WHO)** (WHO, 2020) therefore provides useful contextual information regarding realistic matrices, potential exposure scenarios, and analytical challenges associated with these toxins.

Forensic and incident-investigation contexts

Although EMBRACE does not directly operate within the framework of **the United Nations Secretary-General's Mechanism (UNSGM)** for investigation of alleged biological or chemical weapons use, the analytical strategies described in this deliverable are structured so that their outputs remain compatible with forensic reporting principles relevant to such investigations. In particular, the analytical workflows emphasise qualitative identification, orthogonal analytical confirmation, and transparent reporting of analytical limitations, which are key elements in laboratory reports supporting incident investigations.

Terminology and cross-sector interoperability

To maintain interoperability across CBRN, public-health, and forensic communities, terminology used in this deliverable is aligned where appropriate with commonly used CBRNE definitions, including those compiled in the European CBRNE glossary (Goulart De Medeiros et al, 2022). This alignment is intended to improve cross-sector readability without implying formal adoption of specific regulatory frameworks.

Taken together, these considerations ensure that the analytical strategies developed within EMBRACE remain relevant across multiple operational contexts while remaining feasible within the practical constraints of a single-laboratory analytical programme.

2.2 Analytical philosophy and constraints

2.2.1 Analytical strategy for biotoxin identification

Analytical approaches for the detection and identification of biological toxins present specific challenges due to the diversity of toxin classes, matrices, and analytical technologies required for reliable identification. Biological toxins encompass compounds ranging from small low-molecular-weight molecules to large protein toxins, and these analytes may occur in complex environmental, food, or biological matrices. Consequently, analytical strategies must remain adaptable while still ensuring reproducibility, reliability, and comparability of analytical results.

Within the EMBRACE framework, analytical workflows are therefore organised according to a multi-lane identification strategy, in which complementary analytical techniques provide independent evidence for toxin detection and confirmation. This strategy reflects established practice in forensic

and incident-investigation laboratories, where reliable identification requires the integration of orthogonal analytical methods.

For low-molecular-weight toxins, analytical workflows are built around high-resolution mass spectrometric screening, followed by targeted confirmatory analysis. HRMS-based screening enables broad detection capability across multiple toxin families while also supporting retrospective data analysis. In laboratories with limited HRMS capability, equivalent screening or targeted detection approaches may be implemented using triple-quadrupole LC-MS/MS methods operating in selected-reaction-monitoring (SRM) mode.

For protein toxins, analytical identification relies on a tiered strategy combining immunological screening, functional assays measuring toxin activity, and mass-spectrometric confirmation through peptide analysis. These complementary approaches provide independent analytical evidence for toxin presence and reduce the likelihood of false-positive identification.

The analytical workflows implemented in EMBRACE therefore integrate several complementary analytical lanes:

- HRMS-based screening for low-molecular-weight toxins
- targeted LC-MS/MS confirmation methods
- immunological screening assays for protein toxins
- functional assays measuring toxin activity
- LC-MS/MS-based identification of toxin-specific peptides

This multi-lane strategy ensures that toxin identification can be supported by orthogonal analytical evidence, improving reliability and evidential defensibility in forensic contexts.

2.2.2 Performance-based analytical philosophy

The EMBRACE project adopts a performance-based analytical philosophy, in which analytical methods are evaluated according to their ability to produce reliable analytical outcomes rather than strictly adhering to instrument-specific procedural protocols.

This approach recognises that laboratories may employ different analytical platforms, including immunological assays, chromatographic separation techniques, high-resolution mass spectrometry, or functional bioassays. Rather than prescribing rigid procedural workflows, EMBRACE defines minimum analytical performance expectations, allowing laboratories to implement equivalent analytical strategies using different technologies where necessary.

Performance-based frameworks are widely recognised in international analytical practice. Comparable principles have been adopted in Codex Alimentarius method performance criteria and AOAC standard method performance requirements, which emphasise analytical performance characteristics rather than specific analytical procedures (Codex Alimentarius Commission, 2017; AOAC International, 2016). Such approaches allow analytical methods to evolve alongside technological developments while maintaining comparability of analytical outcomes.

2.2.3 Screening, confirmatory, and field-deployable approaches

Analytical methods used in toxin detection may serve different roles within an incident investigation workflow. In the context of EMBRACE, it is therefore useful to distinguish between screening methods,

confirmatory laboratory analysis, and field-deployable analytical tools, each of which addresses different analytical objectives and evidential requirements.

Screening methods methods are designed to rapidly identify samples that may contain a toxin of interest. Their primary role is to support sample triage and prioritisation, allowing laboratories to focus analytical resources on samples most likely to contain relevant analytes. Screening approaches typically prioritise analytical speed and broad detection capability rather than definitive identification.

Within the EMBRACE analytical framework, screening methods may include immunological assays such as ELISA for selected protein toxins, as well as high-resolution mass-spectrometric screening workflows for low-molecular-weight toxins. HRMS-based screening enables simultaneous detection of multiple toxin classes and provides a flexible discovery layer capable of identifying unexpected toxin analogues. However, screening results are considered indicative rather than confirmatory and therefore require further analytical verification.

Confirmatory laboratory analysis aims to establish compound-specific identity using well-defined analytical criteria and orthogonal analytical evidence. Such confirmation is required before a toxin can be reported as identified in laboratory findings.

For **low-molecular-weight toxins**, confirmatory analysis typically relies on chromatographic separation coupled with tandem mass spectrometric detection. Confirmation may involve evaluation of retention time, precursor and fragment ion masses, diagnostic fragmentation patterns, and comparison with reference standards or spectral libraries.

For **protein toxins**, confirmatory identification often requires a tiered analytical approach combining independent immunological detection, functional activity assays, and peptide-level mass-spectrometric identification. The integration of these complementary analytical dimensions provides strong analytical confidence in toxin identification.

The confirmatory analytical workflows described in this deliverable form the **core focus of Task 3.2,** which aims to develop robust laboratory-based analytical procedures suitable for toxin verification and potential forensic interpretation.

Field-deployable analytical tools represent a complementary category of analytical capability intended for use during incident response or environmental monitoring. Such technologies prioritise portability, rapid response, and operational simplicity, allowing preliminary assessment of samples outside the laboratory environment. However, field-deployable detectors generally do not achieve the analytical specificity required for definitive toxin identification. Their results therefore serve primarily as situational indicators, guiding sampling strategies and prioritisation of laboratory analyses.

Within the EMBRACE project, the **development and evaluation of portable biodetection technologies are addressed in Task 3.3**, which investigates the performance of selected detection platforms under operational conditions. The laboratory-based analytical workflows described in Task 3.2 therefore provide the confirmatory analytical capability required to support reliable toxin identification following initial field screening.

2.2.4 Orthogonality and identification confidence

A central principle guiding analytical workflows within EMBRACE is the use of orthogonal analytical evidence. **Orthogonality refers to the confirmation of analyte identity through independent analytical dimensions that rely on different physical or chemical principles.**

In practical terms, orthogonality may be achieved through combinations of chromatographic separation, mass-spectrometric fragmentation patterns, immunological recognition, or functional activity measurements. The use of multiple independent analytical dimensions reduces the risk of false-positive identification caused by matrix interferences or cross-reactivity.

Within EMBRACE, toxin identification therefore requires at least two independent analytical dimensions before a compound is reported as identified. For low-molecular-weight toxins, orthogonality may be achieved through LC-MS/MS combined with alternative chromatographic mechanisms or chemical derivatisation. For protein toxins, orthogonality is achieved through a tiered strategy combining independent immunological detection and peptide-level LC-MS/MS confirmation.

Analytical results produced within EMBRACE are therefore reported primarily as qualitative identification outcomes, indicating whether a target toxin has been detected with sufficient analytical confidence.

2.2.5 Operational and methodological constraints

Several practical considerations influence the analytical strategies implemented within EMBRACE.

First, analytical activities are conducted within the framework of the **Horizon Europe programme**, which supports research aimed at civil protection, public health preparedness, and forensic investigation capabilities. The analytical methods described in this deliverable are therefore intended to strengthen detection and identification capabilities for biological toxins in environmental, food, and incident-investigation contexts.

Second, analytical work within EMBRACE is primarily conducted within a **single-laboratory configuration,** as WP3 represents the central analytical laboratory within the project. Consequently, the analytical workflows described here are designed to provide reliable toxin identification within a single laboratory environment. At the same time, these methods are intended to remain compatible with broader international analytical networks, including laboratories participating in OPCW-related activities and other CBRN analytical communities. Opportunities for external testing and interlaboratory comparison may arise through these networks, although full formal interlaboratory validation of all analytical workflows is outside the scope of the current project phase.

Third, the analytical strategies developed in EMBRACE aim to remain **as broadly transferable as possible across laboratories with different analytical infrastructures**. While high-resolution mass spectrometry provides the primary screening capability for low-molecular-weight toxins within WP3, equivalent analytical outcomes may be achievable in other laboratories using targeted LC-MS/MS approaches. The methods described in this deliverable are therefore designed, where possible, to remain adaptable to different analytical platforms and laboratory resources.

For **protein toxins**, however, methodological flexibility is inherently more limited. Immunological detection methods depend on the availability of suitable antibodies, which may vary between toxin

targets and between laboratories. As a result, orthogonal identification strategies for protein toxins often require the integration of multiple analytical approaches, including independent immunological assays, functional activity assays, and mass-spectrometric peptide identification. The use of multiple complementary analytical pathways therefore provides both analytical redundancy and improved confidence in toxin identification.

Access to **certified reference materials, toxin standards, and specialised reagents** also varies between toxin classes. Through established collaborations with international partners and specialised suppliers, VER has access to many otherwise difficult-to-obtain materials, including toxin reference standards and immunological reagents required for assay development. Nevertheless, the availability and cost of such materials remain practical factors influencing the pace and scope of analytical development.

Finally, analytical method development within EMBRACE is conducted using **surrogate matrices that represent realistic environmental, food, or biological sample types**, rather than genuine incident samples. These matrices provide controlled experimental conditions for method development and performance evaluation while avoiding the logistical and ethical challenges associated with handling real-world incident samples. However, surrogate matrices may not fully reproduce all characteristics of authentic samples, and this limitation should be considered when interpreting analytical performance.

3 TARGET BIOTOXINS COVERED IN THE FIRST ITERATION

3.1 Overview of selected biotoxins

Definition of the toxin panel

The EMBRACE analytical strategy focuses on a selected panel of biological toxins representing different structural classes, mechanisms of toxicity, and analytical challenges. The objective of this panel is not to provide exhaustive coverage of all known toxins, but rather to represent analytically and operationally relevant toxin families that may be encountered in environmental monitoring, public-health contexts, or incident-investigation scenarios. The selected targets therefore serve as representative analytical challenges for the development of laboratory workflows within Task 3.2.

Molecular and analytical diversity

The toxin panel spans a wide range of molecular sizes and physicochemical properties, including both low-molecular-weight toxins and high-molecular-weight protein toxins (Dorner et al., 2016; Bouaïcha et al., 2019). This diversity ensures that the analytical workflows developed within EMBRACE remain applicable across multiple toxin classes and detection scenarios. The panel therefore includes highly polar alkaloid toxins such as paralytic shellfish toxins, cyclic peptide toxins such as microcystins, fungal mycotoxins such as trichothecenes, plant-derived alkaloids, and large protein toxins including ricin and botulinum neurotoxins (Wiese et al., 2010; Bouaïcha et al., 2019; Rasetti-Escargueil et al., 2024).

Regulatory relevance

Selection of the toxin panel was also guided by international regulatory and non-proliferation frameworks. Certain toxins included in the panel, most notably saxitoxin and ricin, are listed under

Schedule 1 of the Chemical Weapons Convention (CWC) due to their extreme toxicity and potential for misuse (OPCW, 2013). Other toxins, including botulinum neurotoxins, fall within the scope of the **Biological Weapons Convention (BWC)** (Arnon et al., 2001).

The Organisation for Prohibition of Chemical Weapons (OPCW) Scientific Advisory Board (SAB) and its Temporary Working Group on Biotoxins have repeatedly highlighted toxins such as ricin, saxitoxin, and botulinum neurotoxins as particularly relevant for analytical capability development. These discussions typically focus on compounds that combine high toxicity, potential for malicious use, practical availability, and plausible applicability in deliberate misuse scenarios, thereby representing compounds of particular concern from a chemical weapons verification perspective (OPCW, 2023).

Importantly, the scope of the Chemical Weapons Convention is not limited to substances explicitly listed in its schedules. Under the Convention's General Purpose Criterion, any toxic chemical capable of causing death, temporary incapacitation, or permanent harm to humans or animals falls within the scope of the Convention if used for prohibited purposes. Consequently, analytical preparedness must extend beyond scheduled substances to include other biologically derived toxins and rare toxic natural products that could potentially be misused.

Incident preparedness and misuse scenarios

Beyond regulatory considerations, the toxin panel reflects the need to strengthen analytical preparedness for deliberate toxin release scenarios, including potential misuse of biological toxins in criminal or terrorist incidents. Several toxins included in the panel have historically been associated with poisoning incidents or attempted misuse. Well-known examples include the assassination of Bulgarian dissident Georgi Markov in London in 1978 using a ricin-containing pellet, the disrupted ricin production plot in Cologne in 2018, and attempts by the Japanese cult Aum Shinrikyo in the early 1990s to disseminate botulinum toxin. Recent findings on Alexey Navalny's poisoning have illustrated that even unusual or rare toxic natural compounds may appear in deliberate poisoning scenarios. Such cases underline the importance of maintaining broad analytical capabilities capable of identifying both well-known scheduled toxins and unexpected toxic compounds, consistent with the General Purpose Criterion of the CWC.

These cases illustrate that biological toxins remain plausible agents in criminal or terrorist contexts and underline the importance of maintaining robust laboratory capabilities for toxin detection and identification (OPCW, 2023).

The analytical capabilities developed within EMBRACE are also relevant to laboratories participating in investigation frameworks such as the United Nations Secretary-General's Mechanism (UNSGM) for the investigation of alleged use of chemical or biological weapons, where reliable toxin identification in complex or unknown sample matrices may be required (UNODA, 2023).

Environmental and public health relevance

In addition to security-related considerations, the toxin panel includes compounds with clear environmental and public-health relevance. Cyanobacterial toxins and marine biotoxins, for example, are frequently encountered in natural contamination events associated with harmful algal blooms and represent important targets in environmental monitoring and food safety analysis. Including these toxins ensures that the analytical methods developed within EMBRACE remain applicable to both natural contamination scenarios and incident-response contexts (WHO, 2020; Gawankar et al., 2024).

Analytical archetypes and feasibility

From an analytical perspective, several toxins were selected as archetypical representatives of broader toxin families, allowing analytical workflows developed in Task 3.2 to be extended to structurally related compounds. The toxin panel therefore represents a broad spectrum of analytical challenges, including extremely polar small molecules, moderately hydrophobic natural products, cyclic peptides, and large protein toxins (Wiese et al., 2010; Bouaïcha et al., 2019; Beike et al., 2004; Rasetti-Escargueil et al., 2024).

Practical considerations were also taken into account when defining the toxin panel, including the availability of reference materials, analytical standards, and specialised reagents required for method development and verification.

Closing sentence

An overview of the toxin families included in the first iteration of the EMBRACE analytical panel is provided in **Table 1**, while individual toxin profiles are described in Section 3.2. Additional background information on toxin biology, toxicology, and environmental occurrence is provided in **Annex A.**

Table 1. *toxins families currently addressed within the EMBRACE analytical panel.*

Toxin / Toxin family	Molecular class	Primary mechanism	Approx. molecular weight	Primary analytical lane
Ricin	Ribosome-inactivating protein	80S Ribosome inhibitor	65000 Da	Immunoassay → functional assay → LC-MS/MS
Botulinum neurotoxins (BoNT)	Protein neurotoxin	Cleavage of SNARE proteins	150000 Da	Immunoassay → functional assay → LC-MS/MS
Saxitoxin/paralytic shellfish toxins	Marine alkaloid	Na+ channel blocker	300–400 Da	LC-MS/MS
Microcystins / Nodularin	Cyclic peptide	Protein phosphatase inhibition	800–1000 Da	LC-MS/MS
T-2 toxin/Trichothecenes	Mycotoxin	Protein synthesis inhibitor	400–500 Da	LC-MS/MS
Aconitine	Plant alkaloid	Na+ channel activator	645 Da	LC-MS/MS
Strychnine	Plant alkaloid	Glycine receptor antagonist	334 Da	LC-MS/MS

3.2 Individual biotoxin and biotoxin family profiles

This section provides brief profiles of the toxin families included in the EMBRACE analytical panel. The purpose of these descriptions is to summarise the biological origin, toxicological mechanism, and analytical relevance of each toxin group within the context of method development and verification activities carried out in Task 3.2.

The profiles presented here focus primarily on analytical considerations, including structural characteristics and detection challenges relevant to the development of analytical procedures. More detailed background information on toxin biology, toxicology, and environmental occurrence is provided in **Annex A.** The annex will serve as a reference repository for toxin-specific information and will be expanded in subsequent iterations of the deliverable.

3.2.1 Ricin

Ricin belongs to the family of type II ribosome-inactivating proteins (RIP-II toxins) produced by the castor bean plant (Ricinus communis). The toxin is a heterodimeric glycoprotein of approximately 65 kDa, consisting of two polypeptide chains linked by a disulfide bond (Worbs et al., 2015, Audi et al., 2005). The A-chain (~32 kDa) contains the catalytic N-glycosidase activity responsible for depurination of 28S ribosomal RNA, while the B-chain (~34 kDa) functions as a lectin that mediates binding to cell surface carbohydrates and facilitates cellular uptake. The biologically active toxin therefore exists as a disulfide-linked holoenzyme, and both chains may contribute diagnostically relevant peptides in mass-spectrometric identification workflows (Fredriksson et al. 2005, Fredriksson et al., 2015).

The toxic activity of ricin results from N-glycosidase activity that depurinates a specific adenine residue in 28S rRNA, thereby irreversibly inactivating ribosomes and halting protein synthesis (Kalb & Barr, 2009; Audi et al., 2005). Because the catalytic activity of the A-chain is highly efficient, a small number of toxin molecules entering the cytosol can cause profound cellular damage (Audi et al., 2005).

The inhibition of protein synthesis ultimately leads to cell death and tissue damage in affected organs. The systemic manifestations of ricin intoxication depend strongly on the route of exposure (Audi et al., 2005; Worbs et al., 2011). Inhalation exposure primarily affects the respiratory system, causing severe pulmonary inflammation, edema, and progressive respiratory distress (Audi et al., 2005; Worbs et al., 2011). Ingestion leads to gastrointestinal injury, including abdominal pain, vomiting, and hemorrhagic gastroenteritis, which may be followed by systemic toxicity affecting the liver, kidneys, and other organs. Parenteral exposure results in rapid systemic distribution and can lead to multi-organ failure. These diverse clinical manifestations highlight the need for analytical methods capable of detecting ricin and related proteins in a variety of biological and environmental matrices.

From an analytical perspective, ricin should not be regarded as a single uniform analyte (Despeyroux et al., 2000; Sehgal et al., 2011; Fredriksson et al., 2005, 2015). Castor bean varieties may express multiple ricin isoforms, including ricin D and ricin E, with additional heterogeneity arising from cultivar-dependent sequence variation and glycosylation patterns (Despeyroux et al., 2000; Sehgal et al., 2011). Ricin also co-occurs with structurally related proteins such as Ricinus communis agglutinin (RCA120), a lectin with similar sequence motifs but substantially lower toxicity (Roberts et al., 1985; Fredriksson et al., 2015). More broadly, ricin belongs to a wider family of structurally related RIP-II toxins that includes abrin and related plant lectins (Fredriksson et al. 2015, .

These factors introduce important analytical challenges. Immunological detection methods may exhibit cross-reactivity with related lectins, while protein-level mass spectrometric identification must account for isoform diversity and distinguish ricin from closely related homologues. For this reason, robust verification typically relies on multiple orthogonal analytical approaches, including immunological screening, functional assays based on ribosome depurination activity, and confirmatory LC–MS/MS identification of characteristic ricin-derived peptides from both A- and B-chains (Bozza et al., 2015; Fredriksson et al., 2015; Kalb & Barr, 2009; Piquet et al., 2024).

Within EMBRACE, ricin serves as the canonical representative of plant-derived protein toxins, illustrating analytical workflows that integrate immunological, functional, and proteomic identification strategies. Additional background information is provided in **Annex A**.

3.2.2 Botulinum neurotoxins

Botulinum neurotoxins (**BoNTs**) are a family of bacterial protein toxins produced primarily by Clostridium botulinum and related species. Seven major serotypes (BoNT/A–G) have been identified, together with numerous subtypes that differ in amino acid sequence and antigenic properties (Arnon et al., 2001; Dorner et al., 2013; Peck et al., 2017; Smith et al., 2005). The active toxin is produced as a ~150 kDa dichain protein consisting of a catalytic light chain (~50 kDa) and a heavy chain (~100 kDa) responsible for receptor binding and translocation (Montal, 2010; Rummel, 2013). In natural preparations, BoNTs often occur as part of larger toxin complexes associated with accessory proteins, which may influence extraction and detection in complex matrices (Arnon et al., 2001; Dorner et al., 2013).

The toxic activity of BoNT arises from its ability to block neurotransmitter release at neuromuscular junctions. The catalytic light chain functions as a zinc-dependent endopeptidase that cleaves specific components of the SNARE protein complex, including SNAP-25, VAMP/synaptobrevin, and syntaxin (Montal, 2010). Cleavage of these proteins prevents vesicular neurotransmitter release, leading to inhibition of acetylcholine release and the development of flaccid paralysis (Montal, 2010; Rummel, 2013; Boyer et al., 2005).

This highly specific proteolytic activity provides a powerful basis for functional detection methods. In Endopep-type assays, the toxin is first enriched immunologically and then incubated with synthetic peptide substrates mimicking the relevant SNARE target sequence. Serotype-specific cleavage products are subsequently detected by mass spectrometry or immunochemical readout, providing evidence of biologically active toxin and, in many cases, enabling serotype discrimination (Boyer et al., 2005; Kalb et al., 2015; von Berg et al., 2019).

Despite these opportunities, BoNTs present several analytical challenges. The toxins may occur at extremely low concentrations, exhibit significant serotype and subtype diversity, and may be present in partially degraded forms. In natural preparations, BoNTs occur within progenitor toxin complexes that include neurotoxin-associated proteins, such as the non-toxic non-hemagglutinin protein (NTNHA) and hemagglutinin components. These complexes substantially increase the apparent molecular size and influence extraction efficiency, immunological detection, and analytical interpretation in complex matrices. These factors complicate both detection and unambiguous identification (Dorner et al., 2013; Peck et al., 2017; Worbs et al., 2015; Rasetti-Escargueil et al., 2024).

Reliable analytical confirmation therefore typically requires a combination of complementary approaches, including immunological screening methods, functional assays targeting toxin protease activity, and LC–MS/MS identification of toxin-derived peptides following proteolytic digestion (Kalb et al., 2015; Dorner et al., 2013; OPCW, 2023).

Within the EMBRACE framework, BoNTs represent an important example of high-molecular-weight protein toxins requiring multi-layer analytical verification strategies. The analytical approach considered in Task 3.2 therefore adopts a tiered strategy combining immunological screening, activity-based assays such as Endopep-type methods, and confirmatory mass-spectrometric identification of characteristic peptides (Kalb et al., 2015; OPCW, 2023).

Experimental implementation of these approaches is planned for subsequent stages of method development; however, the analytical strategy has already been defined in order to ensure compatibility with the broader EMBRACE toxin detection framework. Additional background information is provided in **Annex A**.

3.2.3 Saxitoxin and paralytic shellfish toxins

Paralytic shellfish toxins (PSTs) are a family of highly polar guanidinium alkaloids produced primarily by marine dinoflagellates, including species of the genera *Alexandrium*, *Gymnodinium*, and *Pyrodinium*, and structurally related toxins are also produced by certain freshwater cyanobacteria (Wiese et al., 2010; Cusick & Sayler, 2013). The toxin family comprises more than fifty structurally related analogues, including saxitoxin and numerous sulfated and carbamoylated derivatives that may occur simultaneously in environmental or food-related samples (Wiese et al., 2010).

Although PST congeners differ structurally, many exhibit comparable biological activity and toxicological properties. Within the framework of the Chemical Weapons Convention, however, only **saxitoxin (STX) is explicitly listed in Schedule 1** (OPCW, 2013). The presence of numerous structurally related congeners therefore introduces an analytical consideration, as detection strategies must account for multiple PST analogues even though regulatory frameworks focus primarily on STX (Harju et al., 2015; Turner et al., 2020).

From an analytical perspective, PSTs present significant challenges due to their extreme polarity and permanent positive charge associated with guanidinium functional groups, which limit retention in conventional reversed-phase chromatographic systems (Cusick & Sayler, 2013; Dell'Aversano et al., 2005). Historically, regulatory monitoring of PSTs in food safety and environmental analysis has relied on oxidative conversion of PSTs to fluorescent derivatives followed by reversed-phase liquid chromatography with fluorescence detection (LC–FLD) (Oshima, 1995; Lawrence et al., 1995; Lawrence et al., 2005). During the oxidation step, structural modifications of the PST molecules reduce their charge density and increase hydrophobicity, producing derivatives that can be effectively separated by reversed-phase chromatography while also generating strong fluorescent signals for detection. Although these methods are well established and widely validated, they involve multiple chemical transformation steps and indirect detection of toxin derivatives (Oshima, 1995; Lawrence et al., 2005; Turner et al., 2020).

More recent analytical approaches increasingly employ hydrophilic interaction chromatography coupled with tandem mass spectrometry (HILIC–LC–MS/MS), which enables direct detection of intact

PST analogues and provides improved selectivity and structural information (Dell'Aversano et al., 2005; Halme et al., 2012; Turner et al., 2020).

Within EMBRACE, saxitoxin (STX) is used as the canonical representative of the PST family. The compound provides a suitable model for the development of analytical workflows targeting extremely polar low-molecular-weight toxins, while allowing evaluation of both traditional and mass-spectrometric detection strategies applicable to related PST analogues.

Additional background information on PST chemistry and environmental occurrence is provided in **Annex A**.

3.2.4 Microcystins

Microcystins (MCs) and nodularin are cyclic peptide toxins produced by freshwater cyanobacteria, particularly species associated with harmful algal blooms in lakes and reservoirs. The microcystin family comprises a large number of structural variants, differing primarily in the amino acid residues incorporated into the cyclic peptide ring. More than two hundred congeners have been described, and multiple variants may occur simultaneously in environmental samples (Namikoshi et al., 1992; Fastner et al., 1999; Bouaïcha et al., 2019).

Microcystins exert their biological activity primarily through inhibition of serine/threonine protein phosphatases PP1 and PP2A, leading to disruption of intracellular signalling pathways and severe cytoskeletal damage. Although these enzymes are widely distributed in mammalian tissues, microcystins exhibit pronounced hepatotoxicity because their cellular uptake is mediated by organic anion transporting polypeptides that are highly expressed in hepatocytes. Transporter-mediated uptake therefore leads to preferential accumulation of the toxins in liver tissue (Bouaïcha et al., 2019).

From an analytical perspective, microcystins represent cyclic peptide natural products of intermediate molecular size (~1000 Da) that are generally well suited for analysis by reversed-phase liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). However, the large number of congeners and their similar fragmentation behaviour present challenges for comprehensive detection and identification (Kaloudis et al., 2013; Turner et al., 2018; Birbeck et al., 2019).

A conserved structural element shared by microcystins and nodularin is the ADDA moiety (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid). Fragment ions derived from the ADDA group are frequently observed in tandem mass spectra and are commonly used as diagnostic indicators for cyanobacterial peptide toxins in LC–MS (Fischer et al., 2001; Guo et al., 2017; Turner et al., 2018).

In environmental monitoring contexts, microcystins are often screened using immunological methods such as ELISA, which detect the ADDA-containing toxin family broadly (Fischer et al., 2001; Turner et al., 2018). Confirmatory identification and congener-specific determination are typically performed using LC–MS/MS methods, which allow discrimination between individual toxin variants.

In addition to reversible enzyme inhibition, certain microcystin congeners can form covalent adducts with protein phosphatases through the reactive Mdha residue present in the toxin structure (Neffling et al., 2010; Cadel-Six et al., 2014; Bouteiller et al., 2022). From an analytical perspective, this behaviour implies that microcystins may occur both as free toxins and as protein-bound forms in biological tissues. While routine analytical workflows typically target the free toxin fraction using LC–MS/MS

following solvent extraction, the detection of toxin–protein adducts after proteolytic digestion has been explored as a complementary strategy for retrospective exposure assessment.

Within EMBRACE, microcystin-LR (MC-LR) is used as the canonical representative of the microcystin toxin family. The compound provides a suitable model for analytical workflows targeting cyclic peptide toxins detectable by reversed-phase LC–MS/MS, while also serving as a reference compound for evaluating detection strategies applicable to structurally related congeners and to the closely related nodularin toxins.

Additional background information on cyanobacterial peptide toxins is provided in **Annex A**.

3.2.5 Plant alkaloid neurotoxins

Plant-derived alkaloid toxins represent a chemically diverse group of naturally occurring small-molecule neurotoxins produced by various plant species. Many of these compounds have historically been associated with accidental poisoning, traditional medicinal use, and deliberate intoxication. From an analytical perspective, alkaloid toxins typically contain basic nitrogen atoms, allowing efficient ionisation in electrospray mass spectrometry and making them well suited for detection using LC–MS/MS methods (Usui et al., 2012; Jaiswal et al., 2013; Lin et al., 2016; Nardin et al., 2016; Zhang et al., 2023).

Within EMBRACE, plant alkaloid toxins are represented by two structurally distinct subclasses: diterpenoid alkaloids, exemplified by aconitine, and indole alkaloids, exemplified by strychnine.

Aconitum alkaloids

Aconitine and related diterpenoid alkaloids are produced by plants of the genus *Aconitum* (monkshood or wolfsbane). These compounds belong to a large family of structurally related alkaloids that occur naturally in several plant species and have historically been associated with both accidental poisoning and deliberate intoxication (Bisset, 1981; Huang et al., 2022; Nyirimigabo, 2015).

The toxic activity of aconitine arises from its interaction with voltage-gated sodium channels, where the toxin stabilises the open state of the channel and prevents normal inactivation. This leads to persistent sodium influx in excitable tissues and results in severe disturbances in cardiac and neuronal electrophysiology, including arrhythmias and neurological symptoms (Nyirimigabo, 2015).

From an analytical perspective, aconitine-type toxins represent moderately hydrophobic alkaloids (~645 Da) that are well retained on reversed-phase liquid chromatography (RP-LC) columns and are readily detectable by LC–MS/MS methods. The molecules contain multiple ester functionalities, and partial hydrolysis can occur during metabolism or sample preparation, producing related compounds such as benzoylaconine and aconine. These metabolites may also serve as relevant analytical targets in biological samples and can provide supporting evidence of exposure (Usui et al., 2012; Wang et al., 2025; Zhang et al., 2025).

Mass spectrometric analysis of aconitine typically produces complex fragmentation patterns, reflecting the highly functionalised polycyclic structure of the molecule. Accurate mass measurements and characteristic fragment ions are therefore important for reliable identification, particularly when analysing complex biological or environmental matrices (Jaiswal et al., 2013; Zhang et al., 2019)..

Within EMBRACE, aconitine is used as a canonical representative of the plant-derived diterpenoid alkaloid toxin class. The compound serves as a model analyte for the development of LC–MS-based workflows targeting moderately hydrophobic natural toxins, complementing the analytical approaches developed for more polar toxins such as paralytic shellfish toxins and for larger peptide toxins such as microcystins.

Indole alkaloids (strychnine-type toxins)

Strychnine is a plant-derived indole alkaloid obtained primarily from the seeds of *Strychnos nux-vomica*. The compound belongs to a group of structurally related alkaloids produced by Strychnos species and has historically been recognised as a classical forensic poison (Duverneuil et al. 2004; Zlotos et al., 2022).

The toxic action of strychnine results from antagonism of glycine receptors in the central nervous system, particularly within the spinal cord and brainstem. By blocking inhibitory neurotransmission mediated by glycine, strychnine causes uncontrolled neuronal excitation, leading to severe muscular convulsions and respiratory failure (Duverneuil et al. 2004; Zlotos et al., 2022).

From an analytical perspective, strychnine represents a basic heterocyclic alkaloid (334 Da) that ionises efficiently in positive electrospray ionisation, typically producing strong [M+H]+ ions. The compound is well retained on reversed-phase LC columns and generates characteristic fragmentation patterns that support reliable identification by LC–MS/MS (Lin et al., 2016; Zhang et al., 2023; Matos et al., 2024).

Strychnine frequently co-occurs with the related indole alkaloid brucine, which possesses similar chemical properties but substantially lower toxicity. Both compounds may be encountered together in plant-derived materials and are therefore often monitored simultaneously in analytical workflows.

Within EMBRACE, strychnine is included as a representative of indole alkaloid neurotoxins and provides a complementary analytical model for basic alkaloid compounds distinct from the diterpenoid alkaloids represented by aconitine.

Additional background information on plant-derived alkaloid toxins is provided in **Annex A**.

3.2.6 T-2-Toxin/Trichothecene mycotoxins

Trichothecenes are a large family of sesquiterpenoid mycotoxins produced by several fungal species, most notably members of the genera *Fusarium*, *Stachybotrys*, and *Myrothecium* (McCormick et al. 2011; Ueno, 1984; Li et al. 2011). These toxins occur primarily as contaminants of agricultural commodities such as cereals and grain products, where they may arise during fungal infection of crops. Trichothecene toxicity is associated with their ability to inhibit protein synthesis inhibiting the ribosome, resulting in rapid cellular toxicity. A defining structural feature of trichothecene toxins is the 12,13-epoxytrichothec-9-ene core, and the presence of the epoxide functionality is essential for biological activity (Bamburg et al., 1968; Ueno, 1984).

Numerous trichothecene variants have been identified, including T-2 toxin, HT-2 toxin, deoxynivalenol, and nivalenol, which differ in their patterns of oxygenation and ester substitution (Li et al., 2011; Ueno, 1984).

From an analytical perspective, trichothecenes represent moderately polar small molecules (molecular mass typically 300–500 Da) that can be analysed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) following appropriate extraction from food or environmental matrices (Li et al., 2011; Nathanail et al., 2015; Pereira et al., 2015). Due to their relatively low molecular mass and thermal stability, trichothecenes can also be detected using gas chromatography–mass spectrometry (GC–MS) following suitable derivatisation.

Analytical detection of trichothecenes can be complicated by the presence of multiple structurally related congeners and by the occurrence of modified or "masked" forms produced during plant metabolism or food processing (McCormick et al., 2015; Kluger et al., 2013; Uhlig et al., 2016). Consequently, analytical workflows often rely on targeted LC–MS/MS methods capable of distinguishing individual congeners through characteristic precursor and fragment ions.

Within EMBRACE, **T-2 toxin** is used as a canonical representative of the trichothecene toxin family. The compound provides a suitable model for analytical workflows targeting fungal small-molecule toxins.

4 ANALYTICAL APPROACHES AND METHODS

4.1 Overview of analytical workflows

The analytical framework developed in Task 3.2 addresses detection and identification of a diverse set of biological toxins comprising both low-molecular-weight compounds and high-molecular-weight protein toxins. Because these analytes differ markedly in chemical structure, physicochemical properties, and biological behaviour, no single analytical technique is sufficient to provide broad screening and decision-grade confirmation across the full toxin panel.

The current strategy is therefore based on a **single integrated analytical workflow** for unknown samples, rather than on separate end-to-end methods for each toxin class. After sample receipt, initial matrix assessment, and assignment to a limited number of broad operational intake classes, samples enter a common analytical pathway that supports broad toxin coverage while allowing branch-specific handling where required by analyte chemistry and evidential needs.

Within this integrated framework, two main analytical branches are used in parallel or in sequence as needed. The first branch addresses **low-molecular-weight toxins**, including paralytic shellfish toxins, microcystins and nodularin, trichothecenes, and plant-derived alkaloid toxins. For these analytes, the workflow is centred on harmonised liquid chromatography–mass spectrometry, combining high-resolution screening with targeted follow-up and orthogonal analytical support where required. This branch is intended to provide broad cross-class screening while preserving the possibility of more selective confirmation for defined analytes.

The second branch addresses **protein toxins**, notably ricin and botulinum neurotoxins. For these toxins, the analytical workflow is inherently more tiered. It combines immunological screening, selective enrichment, functional toxin assays, and peptide-level mass-spectrometric confirmation. Rather than a single universal assay, this branch provides a structured sequence of complementary evidential layers that can be applied according to sample type, matrix complexity, and analytical question.

A central feature of the overall framework is the use of orthogonal evidence. Independent analytical dimensions, such as chromatographic separation, tandem mass spectrometry, immunological recognition, and functional activity measurements, are used to strengthen identification and reduce dependence on any single analytical signal. This is particularly important in a broad screening context, where matrices may be complex and the toxin class may not be known at the point of sample receipt.

The workflow is designed to preserve broad analytical coverage at the initial stage. Where sample amount permits, the low-molecular-weight and protein-toxin branches may be applied in parallel. Where sample amount is limited or case information strongly favours one branch, routing may be prioritised accordingly, while retaining the possibility of subsequent orthogonal follow-up where feasible.

Overall, the analytical framework is intended to provide a flexible but structured basis for laboratory-based verification of biological toxins in diverse incident-related matrices. It is broad enough to

support initial screening of unknown samples, yet sufficiently organised to allow toxin-class-specific confirmation where required.

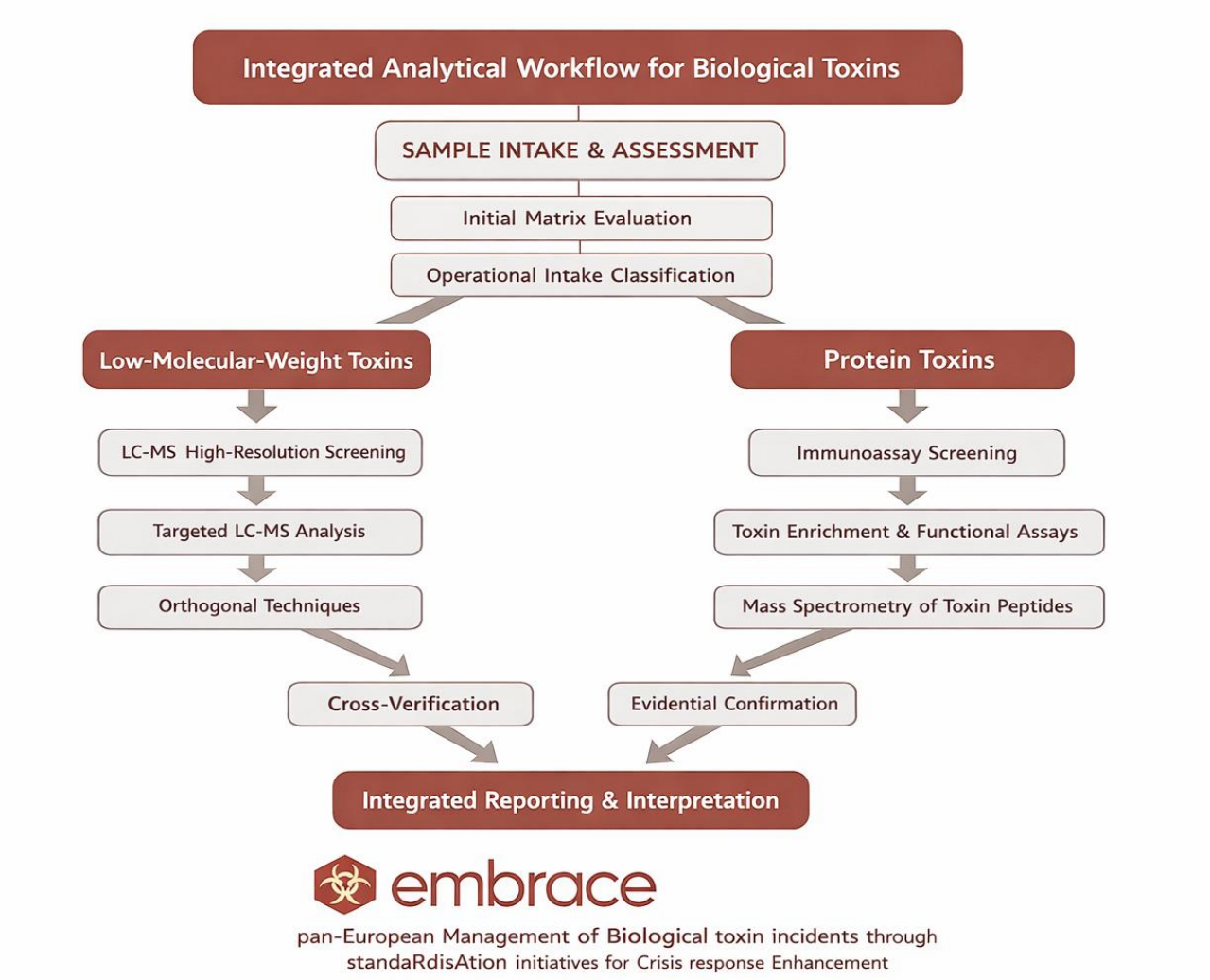

Figure 1. Integrated analytical workflow for biotoxin assessment of unknown samples

4.2 Review of analytical approaches

4.2.1 Ricin

4.2.1.1 Identity, analytical significance, and overall analytical logic

Ricin is a type 2 ribosome-inactivating protein of high forensic and biosecurity relevance because it combines high potency with substantial analytical complexity. The analytical target is not entirely uniform: castor-derived materials may contain ricin together with the closely related *Ricinus communis* agglutinin (RCA120), and ricin itself may occur as different isoforms and glycoforms, which complicates selective detection and interpretation (Roberts et al., 1985; Despeyroux et al., 2000; Sehgal et al., 2011). From an analytical standpoint, no single technique fully addresses all requirements simultaneously, including rapid screening, sensitivity in difficult matrices, demonstration of biological activity, and unambiguous molecular confirmation.

Accordingly, the ricin literature supports a tiered, orthogonal analytical framework in which rapid immunological screening is complemented by functional assays and peptide-level mass-spectrometric confirmation. This overall logic was reinforced by the international EQuATox proficiency exercise, in which the most robust performance was obtained either by combining immunological, functional, and MS-based methods or by applying advanced MS workflows capable of addressing both identity and activity (Worbs et al., 2015a).

4.2.1.2 Sample preparation and enrichment

A defining feature of current ricin analysis is the importance of matrix-aware sample preparation. Relevant matrices span buffered laboratory samples, beverages, milk, meat extracts, environmental powders, fertilizer materials containing castor residues, and biological samples such as serum. Because ricin is often expected at low concentration and in a high-background matrix, front-end processing strongly influences both sensitivity and evidential quality.

For routine immunological screening, sample handling may remain relatively simple and involve extraction, clarification, and dilution into assay-compatible buffer. In the EQuATox ricin proficiency study, for example, milk and meat extract samples could be analysed after spiking into matrix, whereas naturally contaminated fertilizer required extraction into PBS before downstream testing (Worbs et al., 2015a). Participating laboratories also frequently applied preliminary dilution planning or other basic protein-compatible handling steps before committing limited material to more specific assays (Simon et al., 2015).

For confirmatory and high-specificity workflows, however, selective enrichment is typically central. Historically, ricin purification and enrichment relied on the lectin properties of the B-chain. Early affinity-based strategies exploited ricin binding to galactose-containing matrices, first using Sepharose supports with intrinsic carbohydrate-binding properties and later more explicitly derivatised galactose-affinity materials (Tomita et al., 1972; Olsnes et al., 1974). An influential

development was the single-step galactosyl-Sepharose affinity procedure described by Simmons and Russell (1985), in which ricin and the co-expressed agglutinin RCA120 were captured from crude castor bean extracts and differentially eluted by a galactose gradient based on different binding avidity. Purification and reference-material studies continued to employ lactose- or galactose-based affinity steps to isolate ricin and RCA120 from seed extracts before further characterization by electrophoretic and mass-spectrometric methods (Lin & Li, 1980; Worbs et al., 2015b). In parallel, capture methods evolved toward smaller-scale and more MS-compatible enrichment tools, including lactose-immobilized monolithic spin columns and galactose-functionalised magnetic particles (Kanamori-Kataoka et al., 2011; Liu et al., 2011). These approaches linked enrichment directly to downstream LC–MS analysis while preserving the same underlying selectivity principle of B-chain-mediated carbohydrate recognition.

At the same time, many ricin MS-confirmation workflows have relied on immunocapture, followed by digestion and tandem mass-spectrometric analysis of released peptides (Duriez et al., 2008; McGrath et al., 2011; Dupré et al., 2015). Immunoaffinity capture remains highly effective, particularly where validated antibodies are available and the aim is selective peptide confirmation. However, it does not by itself demonstrate preserved lectin functionality of the B-chain and may be more dependent on reagent availability and epitope behaviour.

More recent work has expanded non-antibody affinity capture. Piquet et al. (2024) described an antibody-free approach using **asialofetuin**-coated magnetic beads, again exploiting ricin B-chain lectin binding, but with substantially improved analytical performance compared with older carbohydrate-affinity tools. In that study, ricin recovery after capture was approximately 71%, and the method supported low-ng/mL detection in difficult protein-rich matrices including human serum and pea soup. The same work showed that the capture step tolerated a broad pH range, with peptide detection maintained from pH 4 to pH 10, whereas no useful signal was obtained at pH 3 (Piquet et al., 2024). This is analytically relevant because incident samples may not enter the laboratory in a well-controlled buffer system.

The same study also illustrates an important trend in ricin analysis: acceleration of front-end preparation. By simplifying denaturation and digestion conditions and reducing bead incubation time, the total sample-preparation time was shortened from several hours to about 80 min without abandoning peptide-level confirmation (Piquet et al., 2024). This is attractive for incident-response settings, where the pressure for timely laboratory confirmation is high.

Overall, the literature indicates that sample preparation should be selected according to analytical purpose: simple extraction and dilution for rapid screening; selective enrichment and cleanup for activity-linked or peptide-level confirmation; and, where appropriate, exploitation of B-chain lectin affinity as a robust and historically well-established capture principle.

4.2.1.3 Immunological screening methods

Immunological methods remain the most practical first-line tools for ricin screening because they offer high sensitivity, straightforward implementation, and compatibility with many sample types. Conventional sandwich ELISA formats have been reported with detection limits spanning from ng/mL

down into the low pg/mL range, depending on antibody pair, assay format, and validation design (Griffiths et al., 1986; Leith et al., 1988; Poli et al., 1994; Alderton & Paddle, 1997; Shyu et al., 2002a; Pauly et al., 2009). Multiplexed immunoassay formats have also been demonstrated for ricin in complex matrices (Pauly et al., 2009).

The EQuATox-derived immunological comparison further confirmed the central role of ELISA in ricin screening. Several sandwich ELISA formats successfully identified all or nearly all blinded ricin-containing samples in the proficiency panel, and ELISA methods were among the few approaches that consistently detected the lowest-concentration ricin sample included in the exercise (Simon et al., 2015; Worbs et al., 2015a). This aligns with earlier primary studies showing strong sensitivity in biological samples, foods, and forensic-type materials (Griffiths et al., 1986; Alderton & Paddle, 1997; Pauly et al., 2009).

Portable immunological formats, especially lateral flow assays, provide faster but generally less sensitive screening options. Early colloidal-gold lateral flow assays demonstrated ricin detection within minutes (Shyu et al., 2002b), and later evaluations of field assays showed that some commercial formats can perform well in suspicious powders and environmental samples, although performance varies across products and low-level samples remain challenging (Hodge et al., 2013; Slotved et al., 2014). The proficiency-study-associated work by Simon et al. (2015) similarly showed that some lateral-flow assays were useful for rapid presumptive identification of ricin-containing samples, but they did not match laboratory ELISAs for sensitivity.

The principal analytical limitation of immunometric methods is structural specificity at the forensic level. Because ricin and RCA120 are highly homologous, most immunoassays do not provide unambiguous discrimination between them. Even where preferential ricin- or RCA120-oriented ELISAs are available, clear differentiation still generally requires a second confirmatory layer, usually mass spectrometry (Simon et al., 2015; Worbs et al., 2015a). Immunological methods are therefore best regarded as screening and triage tools, and in some settings as quantitative tools when sufficiently validated, rather than as sole evidence for definitive toxin attribution.

4.2.1.4 Functional assays

Functional assays address a different and highly important question: whether the detected toxin is biologically active. This has implications for hazard assessment, evidential weight, and interpretation of decontamination or inactivation status.

The best-established ricin functional methods are based on A-chain catalytic activity, particularly depurination or N-glycosidase readouts. Adenine release from natural or synthetic substrates has been measured by HPLC, LC–MS, fluorescence, chemiluminescence, and related formats (Hines et al., 2004; Sturm & Schramm, 2009; Bevilacqua et al., 2010). Particularly important for confirmatory practice are methods that combine selective capture with activity readout. Becher et al. (2007) demonstrated immunoaffinity enrichment coupled to LC–MS/MS detection of functional ricin, while Kalb and Barr (2009) described mass-spectrometric detection of both ricin and its enzymatic activity in food and clinical samples. McGrath et al. (2011) further integrated isotope-dilution quantification with enzymatic activity measurement in beverages.

These approaches are analytically valuable because they add biological relevance to the result: they do not merely show that ricin-related protein is present, but that catalytically competent toxin is present. The EQuATox proficiency results support this role. Functional adenine-release assays and cell-based cytotoxicity assays were able to rank ricin, RCA120, and negative samples according to activity in the designated part of the exercise, confirming their value as complementary tools within an orthogonal workflow (Worbs et al., 2015a).

A second functional branch consists of cell-based assays, which assess the integrated outcome of toxin uptake and intracellular action. Examples include GFP-based cytotoxicity assays and real-time impedance-based assays, which have shown ricin detection from culture medium and complex matrices including milk, juice, and food-type samples (Halter et al., 2009; Rasooly & He, 2012; Pauly et al., 2012). These methods are attractive because they reflect combined B-chain and A-chain functionality, but they are typically slower than biochemical activity assays and may have lower selectivity, since other toxic agents can also generate cytotoxic readouts.

A recent report by Liu et al. (2025) proposes that ricin may also exhibit apurinic/apyrimidinic lyase activity in addition to its established N-glycosidase activity, and on that basis describes a fluorescence-based lateral flow assay intended to couple portability with activity-linked readout. This is analytically interesting, but at present it is best regarded as an emerging approach rather than as an established benchmark alongside adenine-release, cytotoxicity, or peptide-MS workflows.

Overall, the literature supports functional assays as a crucial secondary confirmation layer, especially when the analytical question concerns toxin activity, real hazard, or evidential strength beyond simple detection.

4.2.1.5 Peptide-level LC–MS/MS and molecular confirmation

Peptide-level LC–MS/MS has become a principal route to unambiguous ricin confirmation. Its main advantage over immunological screening lies in sequence-level specificity. After capture and proteolysis, ricin-derived peptides can be detected and structurally verified, and appropriately selected peptide panels can distinguish ricin from RCA120 and other related proteins (Fredriksson et al., 2005; Östin et al., 2007; Fredriksson et al., 2015).

Early forensic protein-MS work established the feasibility of identifying ricin in neat toxin preparations and crude castor extracts by mass spectrometry (Fredriksson et al., 2005). Subsequent studies refined this into practical confirmatory workflows using immunocapture or lectin-based enrichment, tryptic digestion, and targeted tandem-MS monitoring. Duriez et al. (2008) demonstrated ricin detection in complex samples by immunocapture and MALDI-TOF MS, while Östin et al. (2007) optimized solvent-assisted trypsin digestion for forensic LC-ESI-MS/MS identification. McGrath et al. (2011) then showed isotope-dilution tandem-MS quantification of ricin in beverages, establishing an important benchmark for structurally specific quantitative confirmation. Dupré et al. (2015) further extended high-resolution targeted MS to multiplexed quantification of protein toxins in human biofluids and food matrices.

The analytical field has since moved toward higher-resolution targeted acquisition, broader peptide coverage, and faster workflows. Piquet et al. (2024) are particularly relevant here. Using asialofetuin-

coated beads and PRM on a high-resolution Orbitrap system, they monitored canonical ricin peptides while also showing that their accelerated workflow could recover additional A-chain and B-chain peptides under data-dependent acquisition. Importantly, their peptide map included ricin-specific peptides not found in agglutinin, preserving the critical function of ricin/RCA120 discrimination. The method reached detection of ricin at 2 ng/mL in buffer and 5 ng/mL in human serum or pea soup as the lowest concentration at which all three monitored peptides gave positive signal, with acceptable repeatability in buffer and soup and somewhat higher variability in serum (Piquet et al., 2024).

Another significant development has been the growing use of non-antibody affinity strategies for MS front-ends. Sugar- or galactose-based enrichment had already been explored earlier, for example using lactose-immobilized monolithic materials and galactose-functionalised magnetic particles (Kanamori-Kataoka et al., 2011; Liu et al., 2011). The asialofetuin strategy of Piquet et al. (2024) can be seen as a further maturation of this concept: a B-chain-directed capture principle that avoids exclusive dependence on proprietary antibodies and may be easier to transfer between laboratories.

In practical terms, peptide-level LC–MS/MS is best understood as the main molecular confirmatory layer for ricin when unambiguous attribution is required, especially for discrimination from RCA120 or for forensic-grade identification in complex matrices.

4.2.1.6 Orthogonality and implications for EMBRACE

The ricin literature consistently supports an orthogonal analytical strategy. Immunometric methods provide the fastest and generally most sensitive route for initial screening. Functional assays address whether catalytically active toxin is present. Peptide-level mass spectrometry provides the clearest route to structurally specific confirmation and discrimination from RCA120. This division of analytical roles is supported not only by individual studies but also by interlaboratory evidence. In the EQuATox ricin proficiency study, the best overall outcomes were obtained either by combining immunological, functional, and MS-based methods or by using advanced MS workflows addressing both identity and activity (Worbs et al., 2015a). Review-level analysis has similarly argued that the ideal ricin assay should combine efficient enrichment, an activity checkpoint, and a selectivity layer that confirms ricin specifically rather than merely detecting a generic toxic signal (Bozza et al., 2015).

For EMBRACE, the practical implication is that ricin should be treated within a tiered workflow rather than assigned to a single-assay solution. A suitable EMBRACE interpretation is: first, screening by sensitive immunological methods, particularly ELISA-type platforms and, where relevant, rapid field-deployable assays for triage; second, activity-linked confirmation by adenine-release or related functional assays when hazard or viability assessment is required; and third, structural confirmation by peptide-level LC–MS/MS or HRMS for definitive ricin attribution and ricin/RCA120 discrimination. This framework is well aligned with forensic logic, with the quality-assurance lessons from EQuATox, and with the broader EMBRACE principle that decision-grade interpretation for complex biotoxins should rely on complementary analytical evidence rather than a single-method claim.

4.2.2 Botulinum neurotoxins

4.2.2.1 Identity, analytical significance, and overall analytical logic

Botulinum neurotoxins (BoNTs) represent one of the most analytically demanding toxin families relevant to forensic and public-health preparedness. This reflects not only their extreme potency, but also the breadth of serotypes and subtypes, the possibility of low toxin burdens in authentic samples, and the diversity of matrices in which they may be encountered, including serum, stool, food matrices, bacterial culture materials, and environmental samples such as soil. An additional complication is that BoNTs may occur either as free 150 kDa neurotoxin or as part of larger progenitor toxin complexes containing associated non-toxic proteins, which can influence extraction behaviour, antibody accessibility, and overall assay performance. Reviews of the field have consistently concluded that these characteristics make BoNTs unsuitable for a single-method analytical solution and instead support a tiered, orthogonal strategy combining enrichment, functional evidence, and structural confirmation (Dorner et al., 2013; Peck et al., 2017; Rasetti-Escargueil et al., 2024).

Accordingly, current analytical best practice treats BoNT detection as a staged verification problem rather than as a one-step assay problem. Immunological methods provide rapid screening and a basis for selective capture, Endopep-type assays provide evidence of catalytically active toxin, and peptide-level LC–MS/MS provides structural confirmation. The resulting analytical logic is therefore inherently multi-layered and matrix-aware.

4.2.2.2 Sample preparation and enrichment

A defining feature of current BoNT analysis is that enrichment is central rather than optional. In realistic matrices, direct analysis is seldom sufficient because expected toxin levels are low while matrix interference is substantial. The prevailing solution in the literature is immunocapture or immunoaffinity enrichment, most commonly using antibodies immobilised on magnetic beads, prior to either functional testing or peptide-level LC–MS/MS. This general approach is found across clinical, food, and environmental applications and has become a core element of both activity-based and structural workflows. In practice, immunocapture serves several analytical purposes simultaneously: it concentrates the toxin, reduces matrix burden, improves sensitivity, and creates a controlled input for downstream functional or mass-spectrometric readout (Boyer et al., 2005; Dorner et al., 2013; Rasetti-Escargueil et al., 2024).

This enrichment-first logic reflects a broader evolution in the field. BoNT analysis has progressively moved away from single-assay detection concepts toward staged, matrix-aware workflows in which sample preparation is treated as an explicit part of the evidential chain. This is particularly important in forensic and verification settings, where poor recovery, matrix-derived false positives or false negatives, and method-dependent subtype bias can materially affect interpretation.

Collaborative studies reinforce the importance of this front-end stage. The EQuATox and EuroBioTox exercises showed that matrix type remains a major determinant of performance, with blank serum proving more challenging than some other matrices and low-level samples producing greater variability than medium-level spiked materials (Worbs et al., 2015; Rasetti-Escargueil et al., 2024).

These findings support the view that BoNT sample preparation should not be treated as a routine pre-analytical step, but as a major determinant of evidential strength.

4.2.2.3 Immunological screening methods

Immunometric methods remain an important component of the BoNT analytical toolbox because they provide relatively rapid, sensitive, and scalable screening while also supplying the antibody reagents needed for enrichment workflows. Sandwich ELISA formats remain the most widely used in vitro methods for toxin detection and quantification, and lateral-flow and microarray variants provide additional options for rapid or multiplexed screening. Interlaboratory work from EQuATox and EuroBioTox showed that several immunological approaches performed well for BoNT/A, BoNT/B, and BoNT/E detection and, in many cases, for quantification as well, particularly when shared reagents and established protocols were used (Sharma et al., 2006; Cheng & Stanker, 2013; Worbs et al., 2015; Rasetti-Escargueil et al., 2024).

At the same time, the literature is clear that immunological methods alone do not normally constitute full forensic-grade confirmation. They demonstrate antigen recognition, but not necessarily catalytic activity, and their performance may vary with serotype or subtype because sequence variation can affect antibody binding and neutralisation. This is an especially important consideration for BoNTs, where subtype-level diversity is analytically consequential (Smith et al., 2005; Peck et al., 2017). Current best practice therefore places immunometric methods primarily in the roles of screening, triage, enrichment, and, where validated, quantification, rather than as stand-alone definitive confirmation tools (Dorner et al., 2013; Rasetti-Escargueil et al., 2024).

4.2.2.4 Functional assays as the leading standard for active toxin detection

The strongest contemporary analytical standard for demonstrating active BoNT is the Endopep-type functional assay. These assays exploit the defining biochemical property of BoNT: the light chain is a zinc-dependent endopeptidase that cleaves specific SNARE proteins, including SNAP-25 and VAMP/synaptobrevin, in a serotype-dependent manner. By incubating captured toxin with synthetic SNARE-derived substrates and measuring the resulting cleavage products, Endopep assays provide direct evidence of catalytically active toxin and also support serotype discrimination through cleavage specificity (Hallis et al., 1996; Boyer et al., 2005; Jones et al., 2008).

This represents a major methodological development relative to the historical dominance of the mouse bioassay. Although the mouse bioassay remains important as a broad-spectrum legacy reference, the literature over the past two decades documents a clear transition toward Endopep-MS and related in vitro functional methods as the preferred route for active toxin confirmation in advanced laboratories. Reviews and collaborative studies describe Endopep-MS as highly sensitive, operationally effective, and increasingly suitable for reference, outbreak-response, and public-health settings, especially when coupled to immunocapture (Dorner et al., 2013; Worbs et al., 2015; Rasetti-Escargueil et al., 2024).

Collaborative proficiency-testing data reinforce this picture. In the first international BoNT proficiency test under EQuATox, and again in the broader EuroBioTox exercise, expert laboratories used combinations of immunological and functional methods across realistic matrices including buffer,

milk, meat extract, serum, and soil. Overall qualitative performance was high, demonstrating both the maturity of the field and the continued importance of combining orthogonal method classes when interpreting challenging samples. These studies also showed that blank serum is analytically more difficult than some other matrices and that low-level BoNT/B samples may be more problematic than comparable BoNT/A samples, again highlighting the need for matrix-aware, multi-method interpretation (Worbs et al., 2015; Rasetti-Escargueil et al., 2024).

Other functional concepts have also been described. For example, Evans et al. (2009) reported an assay that required both functional binding and catalytic activity, illustrating efforts to more closely mimic the biological sequence of intoxication. However, within current analytical practice, Endopep-type assays remain the leading in vitro benchmark for active toxin confirmation.

4.2.2.5 Peptide-level LC–MS/MS and structural confirmation

Functional evidence is only one pillar of the modern BoNT analytical standard. The second pillar is peptide-level LC–MS/MS, which provides structural confirmation of the toxin protein and may support serotype-linked and, in some cases, subtype-aware identification. More broadly, mass-spectrometry-based BoNT strategies have evolved from relatively simple targeted peptide detection toward integrated workflows that combine immunocapture, digestion, proteolytic peptide mapping, and targeted or confirmatory peptide readout as part of a broader identification chain (Boyer et al., 2005; Dorner et al., 2013; Rasetti-Escargueil et al., 2024).

This is important because the analytical question is not always limited to whether an enzymatically active toxin is present. In some settings, there is also a need to determine which toxin form is present, whether the result is consistent with a given serotype or subtype, and whether structural evidence supports or narrows the interpretation generated by screening and functional assays. Peptide-level LC–MS/MS is well suited to this role, but the same interlaboratory literature also shows that its robustness depends strongly on enrichment quality, matrix effects, peptide selection, and operator expertise.

In consequence, peptide-MS is most defensible when embedded in a staged orthogonal workflow rather than deployed as an isolated single-step solution. This conclusion is also consistent with recent proficiency-testing exercises, where LC–MS/MS performed well on blank and medium-level samples but remained less widely implemented than immunological or functional approaches (Rasetti-Escargueil et al., 2024). Thus, while peptide-level LC–MS/MS is analytically powerful, its practical role is as the main structural confirmation layer within a broader evidence framework.

4.2.2.6 QA, orthogonality, and implications for EMBRACE

A notable sign of field maturation is the increasing emphasis on reference materials, collaborative evaluation, and explicit quality-assurance frameworks. EuroBioTox produced recombinant reference materials, including certified BoNT/A material EURM-111, specifically to improve comparability of quantitative results across laboratories. The recent EuroBioTox interlaboratory study showed that quantitative agreement for BoNT/A improved when the shared reference material was used, whereas BoNT/B quantification remained more dispersed in the absence of an equivalent universally applied calibrant (Busschots et al., 2023; Rasetti-Escargueil et al., 2024). Earlier EQuATox and later EuroBioTox

exercises likewise demonstrated the value of structured proficiency testing across multiple matrices, serotypes, concentrations, and method classes, using ISO 13528 principles for study design and data evaluation (Worbs et al., 2015; ISO, 2015; Rasetti-Escargueil et al., 2024).

This direction is consistent with wider international guidance. OPCW Scientific Advisory Board reporting on biotoxins has recommended continued confidence-building exercises, harmonisation of analytical and reporting approaches, and evaluation of methods across different matrices and concentration scenarios (OPCW SAB, 2023). Taken together, these developments show that BoNT analytics has evolved from isolated expert-laboratory practice toward a more collaborative, reference-material-supported, and standards-aware field.

For EMBRACE, the practical implication is that BoNTs are best approached not as a single mature one-step assay problem, but as a multi-layer verification problem in which immunological screening and capture, Endopep-type functional evidence, and peptide-level LC–MS/MS confirmation each contribute distinct analytical value. Within EMBRACE, BoNT is therefore best treated as a defined analytical strategy and supporting toolbox rather than as a fully mature integrated workflow at this stage.

In that sense, BoNT is the clearest example within Task 3.2 of the broader protein neurotoxin problem class: a toxin family that is analytically tractable only through matrix-aware enrichment, active-toxin confirmation, structural confirmation, and explicit orthogonality. That position is well aligned with the current state of the art and with the wider quality-assurance trajectory reflected in EQuATox, EuroBioTox, and OPCW biotoxin guidance.

4.2.3 Paralytic shellfish toxins and saxitoxin-group toxins

4.2.3.1 Identity, structure, occurrence, and analytical significance

Paralytic shellfish toxins (PSTs) comprise a structurally diverse family of saxitoxin-group alkaloids that differ in charge state, sulfation, hydroxylation, and carbamoylation pattern. These features are analytically important because they strongly influence chromatographic behaviour, ionisation efficiency, oxidative response, and susceptibility to interconversion during sample handling (Wiese et al., 2010; Cusick & Sayler, 2013; Harju et al., 2015). Accordingly, the analytical challenge is not simply the detection of “saxitoxin”, but the reliable recognition of a congener system in which multiple native analogues may co-occur and matrix effects may be substantial (Harju et al., 2015; Turner et al., 2020).

4.2.3.2 Core analytical challenges

A central difficulty in PST analysis is that native congeners are highly polar and are not well suited to conventional reversed-phase LC–MS workflows. Their strong hydrophilicity and limited retention on standard RP phases mean that RP-LC generally requires ion-pairing or derivatisation approaches, which may reduce MS compatibility, elevate background, and compromise robustness for confirmatory work (Quilliam, 1996; Quilliam et al., 2001; Dell’Aversano et al., 2005). Native PSTs are therefore, in practical terms, poorly amenable to ordinary RP-LC/MS methods.

Classical LC-FLD approaches circumvent this by converting PSTs to fluorescent derivatives through pre- or post-column oxidation (Oshima et al., 1989; Oshima, 1995; Lawrence et al., 1995). These

methods remain important because they are established, collaborative-study validated, and widely used in shellfish control (Lawrence et al., 2005; Turner et al., 2020). However, they do not measure the native toxins directly. Instead, they infer toxin content from oxidation products, and this creates interpretive limitations: several native precursors may yield identical or overlapping oxidation products, while isomeric congeners are not always straightforward to distinguish after oxidation (Oshima, 1995; Lawrence et al., 2005; Cusick & Sayler, 2013). Thus, although LC-FLD is highly useful for routine monitoring, it is less satisfactory as a stand-alone tool for unambiguous molecular confirmation of native PST profiles.

The fluorescence methods are also operationally demanding. Pre-column oxidation adds manual preparation steps and oxidation-dependent variability, whereas post-column derivatisation requires dedicated instrumentation and stable derivatisation performance throughout the run (Lawrence et al., 1995; Oshima, 1995; Turner et al., 2020). Automated post-column derivatisation can reduce labour, but at the cost of increased instrumental complexity and reduced flexibility.

A further complication is that PST analogues may transform during extraction, storage, or within biological matrices. Such interconversion is well recognised in shellfish and other systems and complicates both calibration strategy and toxin-profile interpretation (Oshima, 1995; Wiese et al., 2010). This is particularly relevant where the native toxin pattern itself carries evidential value.

4.2.3.3 Established analytical approaches

Historically, PST analysis has relied on bioassay and fluorescence-based chromatographic methods. The mouse bioassay was long used as a reference method, but it provides poor chemical specificity, limited sensitivity, and no congener resolution (Cusick & Sayler, 2013; Harju et al., 2015). Its present value is therefore limited.

The main established chemical methods are the AOAC pre-column and post-column oxidation LC-FLD methods (Lawrence et al., 1995; Lawrence et al., 2005; van de Riet et al., 2011). These methods have been foundational in shellfish monitoring and remain highly relevant because of their validation history and broad adoption (Lawrence et al., 2005; Turner et al., 2020). Their strengths lie in maturity and regulatory acceptance. Their limitations are that they are laborious, indirect, and less suited to broader forensic or multisector matrices than to their original shellfish-control context (Oshima, 1995; Turner et al., 2020; Gawankar et al., 2024).

4.2.3.4 HILIC-MS/MS as the principal native-congener approach

The major analytical advance in PST analysis has been the adoption of hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry. HILIC-MS/MS was developed specifically to address the poor reversed-phase behaviour of native PSTs and to permit direct measurement of intact congeners without oxidative derivatisation (Dell'Aversano et al., 2004; Dell'Aversano et al., 2005). By using a polar stationary phase and acetonitrile-rich mobile phases compatible with electrospray ionisation, HILIC enables retention and separation of highly polar PSTs while preserving MS sensitivity and structural specificity (Dell'Aversano et al., 2005; Halme et al., 2012).

This is analytically significant because HILIC-MS/MS observes the native analytes themselves rather than secondary oxidation products. It therefore provides a more direct basis for congener-specific

identification and is better aligned with confirmatory analytical requirements than fluorescence-only workflows (Harju et al., 2015; Turner et al., 2020). Broad congener coverage has been demonstrated for carbamate, decarbamoyl, and N-sulfocarbamoyl toxins, and in some implementations tetrodotoxin is included in the same workflow (Turner et al., 2020).

Single-laboratory and interlaboratory studies have shown that HILIC-MS/MS can provide good sensitivity, linearity, and congener coverage when paired with appropriate clean-up (Boundy et al., 2015; Turner et al., 2015; Turner et al., 2020). Particularly important is the collaborative study by Turner et al. (2020), which demonstrated that UHPLC-HILIC-MS/MS with graphitised carbon SPE clean-up was suitable for determination of multiple PST analogues and TTX across several shellfish matrices, with generally acceptable repeatability, reproducibility, and trueness. This work is important because it places HILIC-MS/MS on a strong multi-laboratory footing.

For EMBRACE purposes, the relevance extends beyond shellfish. Studies in algal material, urine, plasma, freshwater, and mixed food/water matrices show that HILIC-MS-based PST analysis is adaptable to a broad range of matrices, provided that extraction, clean-up, and adsorption control are properly managed (Johnson et al., 2009; Halme et al., 2012; Bragg et al., 2015; Peake et al., 2016; Haddad et al., 2019; Vo Duy et al., 2022; Gawankar et al., 2024).

4.2.3.5 Sample preparation and matrix control

Sample preparation is critical to PST method performance. In shellfish and algal matrices, acidic aqueous extraction is standard, but without further clean-up the resulting extracts are often poorly suited to LC–MS because salts and other coextractives impair both ionisation and chromatography (Turner et al., 2015; Turner et al., 2020). Graphitised carbon SPE has therefore been an important refinement, enabling desalting and improved compatibility with HILIC-MS/MS while preserving broad toxin coverage (Boundy et al., 2015; Turner et al., 2020).

In water and clinical matrices, a variety of SPE approaches have been reported, including weak and strong cation exchange, silica-based, mixed-mode, online SPE, and carbon-based procedures (Johnson et al., 2009; Jansson & Åstot, 2015; Bragg et al., 2015; Xu et al., 2018; Haddad et al., 2019; Vo Duy et al., 2022). The literature does not support a single universal extraction solution; rather, recovery and selectivity remain matrix-dependent because of the extreme polarity, variable charge state, and adsorption behaviour of PSTs (Gawankar et al., 2024). Matrix-specific optimisation is therefore essential.

At trace levels, even container material can matter. Adsorptive losses from aqueous samples have been demonstrated, and polypropylene has been preferred over glass in some workflows to reduce analyte loss (Vo Duy et al., 2022). Such details are operationally minor but analytically important.

4.2.3.6 Screening methods and rapid assays

Immunoassays remain useful as screening tools where rapid triage or broader surveillance is needed. ELISA methods are sensitive, simple, and operationally accessible, and they have been used in environmental and drinking-water surveillance programmes (Cusick & Sayler, 2013; Gawankar et al., 2024). In the EQuATox saxitoxin proficiency test organised by VERIFIN, immunoassays correctly recognised PSP-positive samples, confirming their practical value for screening (Harju et al., 2015).

Their limitations are equally clear. Immunoassays do not resolve individual congeners, cross-reactivity varies by kit and analogue, and they do not provide the structural specificity required for confirmatory identification (Harju et al., 2015; Gawankar et al., 2024). Lateral-flow formats are even less suitable for low-level confirmatory use because of their more limited sensitivity and variable congener response (Harju et al., 2015). They are therefore best placed as preliminary screening methods that trigger confirmatory chromatographic analysis.

4.2.3.7 Evidence from proficiency and collaborative studies

The maturity of PST analytics is supported by both proficiency and collaborative studies. The EQuATox proficiency test is particularly relevant because it examined real algal and mussel matrices rather than only fortified solutions and compared multiple analytical modalities across laboratories (Harju et al., 2015). It showed that laboratories could generally detect STX successfully, but also that immunoassays and MBA lacked congener resolution, the most dilute algal extract produced substantially higher variability, and only a minority of laboratories were able to identify the full analogue set in complex samples (Harju et al., 2015). These observations are directly relevant to forensic and confirmatory work.

The collaborative study of Turner et al. (2020) complements this by showing that a modern HILIC-MS/MS workflow can provide strong interlaboratory performance for a broad PST panel across multiple shellfish species and matrices. Together, these studies show that PST analysis is now sufficiently mature for congener-resolved confirmation, but only when the methods are specifically optimised for PST chemistry and matrix effects.

4.2.3.8 Implications for EMBRACE

The most defensible analytical strategy is a tiered and orthogonal one. Immunological assays may be useful for rapid screening, but confirmatory analysis should rely on native-congener LC–MS/MS, with HILIC as the default chromatographic mode. Legacy LC-FLD methods remain valuable for interoperability with established monitoring practice and as comparator methods, but they should not be treated as the preferred sole confirmatory layer for forensic-style applications because they measure oxidation products rather than intact analytes and are constrained by precursor-product ambiguity, isomerism, and workflow burden (Oshima, 1995; Lawrence et al., 2005; Turner et al., 2020).

In practical terms, this supports a strategy based on matrix-appropriate extraction and clean-up, native-congener separation by HILIC-MS/MS as the principal confirmation route, optional LC-FLD for comparability with established practice, and careful interpretation of analogue patterns in light of possible interconversion and matrix-driven bias.

Overall, the literature supports a clear analytical conclusion. Native PSTs are poorly suited to conventional RP-LC/MS, oxidation-based fluorescence methods remain important but indirect and laborious, and HILIC-MS/MS has emerged as the most appropriate platform for congener-resolved confirmation across relevant matrices (Dell'Aversano et al., 2005; Harju et al., 2015; Turner et al., 2020; Gawankar et al., 2024).

4.2.4 Aconitum alkaloids and strychnine-type alkaloids: analytical methodology

4.2.4.1 Identity, structure, occurrence, and analytical significance

The plant alkaloid toxins considered here are the aconitum diterpenoid alkaloids and the strychnine-type indole alkaloids, especially strychnine and brucine. Both groups are well suited to LC–MS analysis because they ionise efficiently in positive electrospray and generally yield informative MS/MS spectra. Their analytical relevance arises from toxicological importance, occurrence in botanical and biological matrices, and the need to distinguish parent toxins from related congeners, hydrolysis products, or metabolites.

Aconitum alkaloids are characteristic constituents of *Aconitum* species. The principal toxic markers are the diester diterpenoid alkaloids (DDAs), notably aconitine, mesaconitine, and hypaconitine, together with other toxic congeners such as yunaconitine, indaconitine, 10-hydroxymesaconitine, deoxyaconitine, crassicauline A, foresaconitine, 3-acetylaconitine, and bulleyaconitine A (Lai et al., 2006; Chung et al., 2012; Huang et al., 2022; Zhang et al., 2025; Wang et al., 2025). The corresponding monoester diterpenoid alkaloids (MDAs), including benzoylaconine, benzoylmesaconine, and benzoylhypaconine, occur naturally and also arise through hydrolysis during processing or degradation (Usui et al., 2012; Zhang et al., 2025; Wang et al., 2025). Less esterified alcohol amine-type alkaloids, such as bullatine A, bullatine B, and talatisamine, are generally less toxic but can still contribute to toxicological interpretation and plant-source profiling (Wang et al., 2025).

Structurally, aconitum alkaloids are polycyclic norditerpenoid bases with multiple oxygen substituents and variable esterification. The most toxic DDAs characteristically bear an 8-*O*-acyl substituent and a 14-*O*-aroyl substituent, commonly the 8-*O*-acetyl and 14-*O*-benzoyl pattern seen in aconitine, mesaconitine, and hypaconitine (Bisset, 1981; Friese et al., 1997; Huang et al., 2022). Progressive hydrolysis converts these to MDAs and subsequently to less esterified alcohol amine-type alkaloids. This structural series has direct analytical consequences, because it spans compounds of differing polarity, lipophilicity, chromatographic retention, extraction behaviour, and stability (Usui et al., 2012; Zhang et al., 2025; Wang et al., 2025).

Aconitum intoxications are therefore best approached analytically as multi-analyte events rather than as single-compound exposures. Early forensic and clinical LC–MS/MS studies already demonstrated distribution of aconitine, mesaconitine, hypaconitine, and related alkaloids across blood, urine, and tissues in fatal poisoning cases (Beike et al., 2004; Usui et al., 2012; Niitsu et al., 2013). Later work showed that additional markers, including yunaconitine, crassicauline A, bulleyaconitine A, and alcohol amine-type alkaloids, may also be relevant in diagnosis and interpretation (Lai et al., 2006; Chung et al., 2012; Zhang et al., 2025; Wang et al., 2025).

The strychnine-type alkaloids are analytically narrower in scope. The main compounds are strychnine and brucine, the principal toxic alkaloids of *Strychnos nux-vomica* and related materials, together with strychnine *N*-oxide and brucine *N*-oxide as relevant metabolites (Lin et al., 2015). These are rigid, highly functionalised indole-derived cage alkaloids. Brucine is the more methoxylated analogue of strychnine. Both compounds protonate readily in positive ESI and generate characteristic product-ion

spectra, which has made them suitable targets in pharmacokinetic, forensic, and broader multianalyte toxicology methods (Lin et al., 2015; Zhang et al., 2023; Matos et al., 2024).

Analytically, the two groups differ mainly in breadth. Aconitum alkaloids are best treated as a structurally diverse target family comprising parent toxins, hydrolysis products, and less common congeners. Strychnine and brucine are more focused targets, typically measured together with a limited number of metabolites or incorporated into broader toxicology panels.

4.2.5 Sample preparation in current practice

Sample preparation is determined mainly by matrix type and target scope. For aconitum alkaloids in blood and urine, the strongest current precedents are mixed-mode cation-exchange SPE and supported liquid extraction (SLE). Usui et al. (2012) used Oasis MCX for simultaneous determination of 11 aconitum alkaloids in serum and urine. Wang et al. (2025) applied an Oasis PRIME MCX µElution workflow to 19 aconitum alkaloids in whole blood and urine, using acidification, sorbent loading, acidic and organic washing, and basic methanolic elution. Zhang et al. (2025) developed an SLE-UPLC-MS/MS method for 14 aconitum alkaloids in blood and urine, using buffered loading onto diatomaceous-earth cartridges followed by ethyl acetate elution. Both strategies gave acceptable recoveries and matrix effects and are clearly compatible with routine multi-analyte LC–MS/MS.

Older methods also used liquid–liquid extraction, typically after alkalinisation, for aconitine-type alkaloids in biological matrices (Beike et al., 2004; Lai et al., 2006; Wang et al., 2007). These approaches remain workable, but SPE- and SLE-based methods now provide a stronger basis for broader-panel analysis.

For aconitum alkaloids in herbal materials, preparation is usually based on solvent extraction, commonly with methanol-, ethanol-, or acidified aqueous-organic mixtures, sometimes assisted by ultrasound or related extraction steps (Liu et al., 2006; Song et al., 2012; Huang et al., 2022). In more specialised direct-analysis or imaging workflows, sample preparation may be reduced to grinding, powder handling, or tissue-section preparation rather than conventional extract cleanup (Zhu et al., 2011; Tan et al., 2022; Zhou et al., 2023).

For strychnine and brucine in plasma, the literature supports relatively simple preparation. Lin et al. (2015) used methanolic protein precipitation, followed by evaporation and reconstitution, for simultaneous measurement of strychnine, brucine, and their *N*-oxides in rat plasma. In broader toxicology workflows, protein precipitation with acetonitrile can also be sufficient, depending on the matrix and analyte panel (Matos et al., 2024).

For mixed toxic-alkaloid forensic blood analysis, simpler solvent extraction can also be effective. Zhang et al. (2023) used ether extraction under alkaline conditions for simultaneous analysis of brucine, strychnine, aconitine, mesaconitine, and hypaconitine in blood by UPLC-MRM-IDA-EPI.

Overall, current practice supports three main conclusions: MCX-type SPE and SLE are the most mature approaches for aconitum alkaloids in biological matrices; protein precipitation is adequate for strychnine/brucine plasma work in appropriate settings; and solvent extraction remains standard for plant materials.

4.2.5.1 Analytical methods used: overview

The principal analytical platform for both analyte groups is LC–MS/MS in positive ESI mode. For aconitum alkaloids, earlier analytical approaches included HPLC-UV/DAD and capillary electrophoresis. HPLC-UV methods were used for aconitine-type alkaloids in both plant extracts and biological samples (Wang et al., 2004; Xie et al., 2005; Csupor et al., 2009). Capillary electrophoresis was also shown to separate aconitine, mesaconitine, and hypaconitine in herbal preparations (Li et al., 2004; Zhao et al., 2004; Qi et al., 2009). These methods established analytical tractability but are now secondary to LC–MS-based approaches.

Current aconitum analysis is centred on UPLC/UHPLC–MS/MS, usually with triple-quadrupole instruments operated in MRM mode. This has been applied in clinical, forensic, pharmacokinetic, and tissue-distribution studies, with analyte panels ranging from the classical three major DDAs to 19 alkaloids covering DDAs, MDAs, and alcohol amine-type compounds (Lai et al., 2006; Chung et al., 2012; Usui et al., 2012; Zhang et al., 2025; Wang et al., 2025).

High-resolution MS has a complementary role, especially in herbal profiling and broader structural work. QTOF- and Orbitrap-based studies have been used to characterise aconitum alkaloids in botanical materials, tissues, and poisoning-related metabolomic contexts (Jaiswal et al., 2013; Zhang et al., 2019).

For strychnine and brucine, the modern literature is likewise centred on LC–MS/MS. Lin et al. (2015) developed a dedicated LC–MS/MS assay for strychnine, brucine, strychnine *N*-oxide, and brucine *N*-oxide in rat plasma. Zhang et al. (2023) included strychnine and brucine in a broader toxic-alkaloid assay using QTRAP-based MRM-IDA-EPI. Strychnine also appears in broader multiresidue toxicology methods, including post-mortem pesticide and toxicant panels (Matos et al., 2024).

For herbal alkaloid profiling, HRMS-based targeted and untargeted approaches have also been used. Nardin et al. (2016) showed that online SPE coupled to Q-Orbitrap HRMS supports broad alkaloid profiling in herbal extracts, including validated measurement of strychnine. Bonnet et al. (2022) used HPLC-ESI-QTOF molecular networking to characterise alkaloid diversity in *Strychnos* extracts.

In summary, the methods hierarchy is straightforward: LC–MS/MS is the routine analytical core, HRMS extends scope for profiling and suspect analysis, and older optical or electrophoretic methods remain relevant mainly as historical or complementary precedents.

4.2.5.2 Screening methods and class-oriented approaches

For these analytes, screening is most often implemented as broad targeted LC–MS/MS or broader HRMS alkaloid profiling, rather than as immunochemical or dedicated field-based rapid tests.

For aconitum alkaloids, broad targeted LC–MS/MS panels already function as effective first-line screening tools when the analyte space is defined. Serum, urine, blood, and tissue studies have shown that screening multiple aconitum alkaloids at once is more informative than targeting aconitine alone (Usui et al., 2012; Zhang et al., 2025; Wang et al., 2025). A second, more specialised screening line is represented by direct-analysis or ambient-MS methods such as DART-MS, oEESI-MS, and related formats for rapid herbal-material examination (Qiu et al., 2007; Qiu et al., 2023).

For strychnine and brucine, screening is usually embedded in broader toxicological LC–MS workflows rather than treated as a distinct analytical class. QTRAP MRM-IDA-EPI adds an additional spectral-confirmation layer within the same LC–MS/MS run and is one useful example of this approach (Zhang et al., 2023). HRMS-based alkaloid profiling has also been used for *Strychnos* extracts, but primarily in phytochemical rather than routine toxicological settings (Nardin et al., 2016; Bonnet et al., 2022).

Overall, the literature supports only a limited screening section here. The main established approach is broad targeted LC–MS/MS, with HRMS and ambient-MS methods providing additional precedents in botanical-material analysis.

4.2.5.3 Current LC–MS scientific standard

The current scientific standard for both groups is RP-LC–ESI(+)-MS/MS, most commonly on triple-quadrupole instruments in MRM mode.

For aconitum alkaloids, the strongest current standard is a multi-analyte LC–MS/MS method including the major DDAs and, where possible, relevant MDAs and additional toxic congeners. The progression from focused three-analyte methods to 11-, 14-, and 19-analyte panels shows that broader coverage is analytically feasible and toxicologically useful (Beike et al., 2004; Usui et al., 2012; Zhang et al., 2025; Wang et al., 2025). Current best practice therefore combines positive ESI, panel-based MRM acquisition, and appropriate cleanup, typically MCX SPE or SLE for blood and urine.

For strychnine and brucine, the same general platform applies, but with a narrower analyte scope. The most established form is a dedicated LC–MS/MS assay for strychnine, brucine, and their *N*-oxide metabolites where required (Lin et al., 2015). In forensic multianalyte toxicology, these compounds can also be incorporated into larger panels without change in the underlying analytical logic (Zhang et al., 2023; Matos et al., 2024).

HRMS is not the routine quantitative default, but it is a justified complementary platform where broader profiling, retrospective review, or plant-material characterisation is needed (Nardin et al., 2016; Bonnet et al., 2022; Zhang et al., 2019).

Accordingly, the current LC–MS scientific standard is best defined as positive-ion RP-UHPLC/UPLC–MS/MS for routine confirmation, with HRMS as an adjunct for broader structural coverage.

4.2.5.4 Functional and orthogonal approaches

The literature provides some orthogonal or complementary precedents, but these are limited and analyte-dependent.

For aconitum alkaloids, the clearest non-RP-LC–MS precedent is capillary electrophoresis, which has been applied to aconitine, mesaconitine, and hypaconitine in herbal materials (Li et al., 2004; Zhao et al., 2006; Qi et al., 2006). HPLC-UV/DAD also exists as an older complementary approach (Wang et al., 2004; Xie et al., 2007; Csupor et al., 2009). More specialised alternatives include DART-MS, oEESI/H-oEESI-MS, DESI-MSI/HPTLC-DESI-MSI, and SERS, mainly for rapid herbal analysis, direct profiling, or spatially resolved studies rather than routine forensic confirmation (Qiu et al., 2007; Zhu et al., 2011; Zhou et al., 2023; Qiu et al., 2023; Wang et al., 2024).

For strychnine and brucine, truly orthogonal precedents are fewer. The literature remains dominated by LC–MS/MS and HRMS-based alkaloid profiling, including QTRAP MRM-IDA-EPI and Orbitrap/QTOF profiling approaches (Lin et al., 2015; Nardin et al., 2016; Bonnet et al., 2022; Zhang et al., 2023). Non-MS orthogonal methods are not comparably established in the recent literature for this narrower analyte group.

No clearly established functional assay family analogous to those used for some protein toxins was identified in the analytical literature for either group. Orthogonality here is therefore primarily separation-based or instrumental, not functional.

4.2.5.5 Implications for EMBRACE

For EMBRACE, the literature supports a straightforward analytical position. First, both aconitum alkaloids and strychnine-type alkaloids are well suited to RP-LC–ESI(+)-MS/MS as the primary confirmatory platform. These analytes are MS-friendly, give strong positive-ion response, and provide informative fragmentation. The main technical question is therefore not whether LC–MS is appropriate, but how broad the analyte panel should be and how matrix-specific preparation should be organised.

Second, for aconitum alkaloids, the literature supports panel-based analysis rather than a narrow single-analyte approach. At minimum, the core panel should include aconitine, mesaconitine, and hypaconitine, with extension to MDAs and selected additional toxic congeners where broader evidential coverage is required (Usui et al., 2012; Zhang et al., 2025; Wang et al., 2025).

Third, for sample preparation, the best-supported choices are MCX SPE or validated SLE for aconitum alkaloids in blood and urine, protein precipitation for strychnine/brucine plasma work where appropriate, and solvent extraction for plant materials, with additional cleanup only as needed.

Fourth, if a complementary non-RP approach is required, the literature offers stronger precedents for aconitum alkaloids than for strychnine-type alkaloids, particularly through capillary electrophoresis and selected direct-analysis techniques.

Overall, the literature supports an LC–MS-centred workflow with matrix-appropriate preparation, broader aconitum panel coverage, and optional HRMS or secondary orthogonal methods where added structural or evidential scope is required.

4.2.6 Trichothecenes

4.2.6.1 Identity, structure, occurrence, and analytical significance

Trichothecenes are a structurally related family of sesquiterpenoid mycotoxins defined by a tricyclic 12,13-epoxytrichothec-9-ene core. This epoxide is central both to toxic action and to class identity, and it therefore matters analytically as well as toxicologically (Ueno, 1984; Shifrin & Anderson, 1999). Within this shared scaffold, structural diversity arises through oxygenation state, esterification pattern, and substitution at several positions, especially C-3, C-4, C-8, and C-15. On this basis, trichothecenes are commonly grouped into types A, B, C, and D, of which type A and type B are the most relevant for

cereal contamination, food/feed monitoring, and most current LC-MS workflows (Bamburg et al., 1968; Ueno, 1984; Li et al., 2011).

Type A trichothecenes include T-2 toxin, HT-2 toxin, diacetoxyscirpenol, neosolaniol, 15-acetoxyscirpenol, T-2 triol, and T-2 tetraol. They lack the C-8 keto group found in type B compounds and often contain ester substituents that increase hydrophobicity and alter fragmentation behaviour. Type B trichothecenes include deoxynivalenol (DON), nivalenol (NIV), fusarenon-X, and the acetylated DON derivatives 3-AcDON and 15-AcDON, together with biologically or environmentally formed products such as deepoxy-DON, DON-3-glucoside, 3-epi-DON, and 3-keto-DON (Yoshizawa et al., 1980; Swanson et al., 1987; McCormick et al., 2015; Wu et al., 2023). For analytical purposes, this diversity means that trichothecene analysis is not a single-target problem but a family-level problem involving analytes with substantially different physicochemical behaviour.

That chemistry has direct implications for analytical design. T-2 toxin, with multiple acetyl groups and an isovaleryl ester, is more hydrophobic than HT-2, which in turn is less polar than T-2 triol or T-2 tetraol. DON and NIV are appreciably more polar than T-2-type toxins, while glucosylated or further transformed metabolites can be more polar still. As a result, extraction efficiency, SPE retention, chromatographic retention, ionisation mode preference, source behaviour, and matrix effects vary substantially across the class (Klötzel et al., 2005; Sulyok et al., 2006; Nathanail et al., 2015; Wu et al., 2023). This is one reason why broad trichothecene methods are usually compromise methods, and why some workflows separate class members by analytical purpose rather than attempting a single maximally inclusive method.

A second important analytical feature is that some trichothecenes are isomeric or very closely related structurally, so exact mass alone may be insufficient for confident assignment. A classic example is 3-AcDON versus 15-AcDON, which share the same elemental composition and similar MS/MS behaviour and therefore generally require chromatographic resolution for confident differentiation. Similarly, in-source transformations and shared fragments can complicate assignment of DON, DON-3-glucoside, and related products if method conditions are not carefully controlled (Tamura et al., 2015; Wu et al., 2023). The analytical problem is therefore not simply detecting a toxin class, but distinguishing among structurally similar congeners and metabolites in the presence of matrix interferences.

A third important point is that the class is analytically open-ended. Beyond the well-known parent toxins, trichothecenes undergo hydrolysis, deacetylation, hydroxylation, deepoxidation, glucosylation, sulfation, and thiol conjugation in plants, fungi, animals, and environmental systems. T-2 toxin yields HT-2 toxin, T-2 triol, T-2 tetraol, 3′-hydroxy T-2, 3′-hydroxy HT-2, and deepoxy metabolites *in vivo*, depending on species and matrix (Yoshizawa et al., 1980; Corley et al., 1985; Corley et al., 1986; Visconti & Mirocha, 1985). DON likewise forms glucosides and a growing set of sulfur-linked conjugates, including glutathione, cysteinylglycine, cysteine, and *N*-acetylcysteine adducts, some of which can occur naturally in grain (Kluger et al., 2013; Kluger et al., 2015; Stanic et al., 2015; Stanic et al., 2016a; Stanic et al., 2016b; Uhlig et al., 2016). From an analytical standpoint, this means that standard-based targeted methods are essential but inherently incomplete.

Occurrence patterns also shape analytical priorities. T-2 and HT-2 are especially associated with oats and barley in cooler northern European conditions and are linked particularly with *Fusarium langsethiae* and *Fusarium sporotrichioides*, whereas DON, NIV, and their acetylated forms are widely associated with *Fusarium* head blight complexes in wheat, maize, and barley (Edwards et al., 2012; Hofgaard et al., 2016; Hietaniemi et al., 2016; Schöneberg et al., 2018; Meyer et al., 2021). In real samples, multiple trichothecenes and modified forms often co-occur, so single-analyte methods are usually less representative of field reality than multi-analyte workflows (Sulyok et al., 2006; Kovalsky et al., 2016).

Overall, the chemistry argues for a layered analytical framework. The shared epoxide-containing core supports class-oriented thinking, but analyte-specific diversity strongly affects extraction, chromatographic behaviour, ionisation, fragmentation, and detectability. For EMBRACE, this means that trichothecene analysis should be framed not as detection of one toxin, but as characterization of a structurally diverse family comprising parent toxins, known metabolites, and potentially under-characterised transformed analogues.

4.2.6.2 Sample preparation in current practice

Current sample preparation for trichothecenes is driven primarily by matrix complexity and secondarily by the breadth of the analyte panel. In cereal grains and cereal-based foods, the basic extraction logic is still solvent extraction with aqueous organic mixtures, most commonly acetonitrile/water or methanol/water. For broad cereal methods, acetonitrile/water mixtures, often around 79:20:1 acetonitrile/water/acetic acid or similar, have been used effectively, especially in LC-MS/MS methods targeting multiple *Fusarium* toxins (Sulyok et al., 2006; Kolawole et al., 2021). Other methods use methanol/water systems, particularly where better recovery of polar analytes or modified forms is needed (Wu et al., 2023). The choice is not trivial: salting-out or strongly organic conditions can compromise recovery of more polar analytes such as DON-3-glucoside, whereas simple methanolic extraction may be less efficient for hydrophobic analytes or may not sufficiently suppress matrix burden (Wu et al., 2023).

For cereals and feeds, four sample-preparation philosophies dominate: dilute-and-shoot, QuEChERS or simplified QuEChERS-like extraction, SPE or multifunctional cartridge cleanup, and immunoaffinity cleanup for selected analytes. Dilute-and-shoot is attractive for high-throughput multiclass work because it avoids selective cleanup that might exclude relevant metabolites, and it has been used successfully for broad multi-mycotoxin methods in wheat and maize (Sulyok et al., 2006; Malachová et al., 2014; Sulyok et al., 2020). Its drawback is greater matrix burden and therefore higher susceptibility to ion suppression, contamination, and sometimes higher limits of quantification. It is most defensible when the instrument is sufficiently robust and the analytical aim is broad coverage rather than the lowest possible LOQ for every analyte.

QuEChERS-type workflows have become common because they provide a practical middle ground between crude extraction and laborious cleanup. In trichothecene work they are particularly useful for cereal products and broader multiclass toxin panels, and modified or simplified versions without full dispersive cleanup have also been used when analysts want to preserve analyte breadth while still reducing matrix effects to some extent (Annunziata et al., 2017; De Colli et al., 2020; Kim et al., 2022).

However, strongly polar analytes can still be problematic in some QuEChERS workflows, and method-specific validation is essential rather than assuming generic performance across the class.

SPE and related cartridge cleanup remain important where lower LOQs or cleaner extracts are needed. HLB, C18, MycoSep, Bond Elut Mycotoxin, and other sorbents have all been used in trichothecene methods, often as part of multiclass cereal workflows (Klötzel et al., 2005; Gottschalk et al., 2007; Lattanzio et al., 2011; Meyer et al., 2021). Their main strength is improved cleanliness and sensitivity. Their main weakness is potential selectivity bias, especially if the method aims to capture both hydrophobic T-2-like analytes and more polar DON-related metabolites, including conjugates. In practice, the more chemically diverse the target list, the less universally suitable a selective cleanup becomes.

For biological matrices, sample preparation becomes more matrix-specific. In plasma, simple protein precipitation with acetonitrile or acidified acetonitrile can be sufficient for many analytes, sometimes with phospholipid-removal steps added for avian plasma or other more demanding matrices (De Baere et al., 2011; Lauwers et al., 2019). In urine, liquid–liquid extraction has often been preferred over dilute-and-shoot when practical sensitivity and reduced matrix effects are desired, though pH adjustment may be needed depending on the analytes targeted (Song et al., 2013; Lauwers et al., 2019). In faeces and excreta, the matrix is much more challenging, and double extraction, phospholipid cleanup, or matrix-tailored extraction protocols may be required if the analyte panel spans multiple mycotoxin families or includes both free toxins and metabolites (Lauwers et al., 2019).

For modified and conjugated trichothecenes, sample preparation can be analytically decisive. The work of Kluger and co-workers and Uhlig and co-workers shows that sulfur-linked DON conjugates and other masked or transformed forms can be present at lower abundance and may require HRMS-compatible extraction that preserves them without overly selective cleanup (Kluger et al., 2013; Kluger et al., 2015; Uhlig et al., 2016). In such cases, overly aggressive cleanup can defeat the point of broader structural interrogation.

Overall, trichothecene sample preparation is best treated as a controlled trade-off among coverage, cleanliness, sensitivity, and practicality. For EMBRACE, the most realistic approach is not a single universal extraction for all matrices, but a matrix-grouped strategy: streamlined aqueous-organic extraction for cereals and powders; modified QuEChERS or dilute-and-shoot for broader screening workflows; and tailored precipitation- or LLE-based methods for biological matrices.

4.2.6.3 Analytical methods used

The analytical families most relevant to trichothecenes are immunochemical methods, chromatographic methods with optical detection, targeted LC-MS/MS, and HRMS-based targeted or broader screening methods. In modern practice, the centre of gravity is clearly with LC-MS, but older and simpler methods remain useful for understanding current workflows.

Immunochemical methods such as ELISA, lateral-flow devices, and related rapid screening tools remain useful where high throughput and operational simplicity are more important than structural detail. They are widely used for food and feed screening, especially for DON and for the combined T-2/HT-2 question, but they depend strongly on antibody cross-reactivity and can over- or under-

respond depending on metabolite profile and matrix (Aamot et al., 2013; Oplatowska-Stachowiak et al., 2017; Lippolis et al., 2019). For type A trichothecenes in particular, antibody cross-reactivity with HT-2, T-2 metabolites, or glucosylated forms can be either advantageous or problematic depending on the analytical aim. These methods are therefore useful as screening tools but generally weaker as stand-alone confirmatory methods.

Older chromatographic methods such as GC-MS, GC-MS/MS, HPLC-UV, and HPLC-FLD played an important historical role. GC-based methods can be sensitive but often require derivatisation because trichothecenes are insufficiently volatile in native form, which adds complexity and potential error. HPLC-UV and HPLC-FLD have also been used, but limited chromophore strength and the need for derivatisation in fluorescence-based methods restrict their attractiveness for current broad routine work (Soleimany et al., 2012; Trebstein et al., 2008; Pereira et al., 2015). These methods are now secondary to LC-MS except in specific regulatory or legacy settings.

LC-MS/MS is the current mainstay for confirmatory targeted trichothecene analysis. Triple-quadrupole instruments operated in SRM/MRM mode provide the best established balance of selectivity, quantitative performance, speed, and robustness for known analytes. They are especially effective where the analyte list is defined and standards are available, for example for DON, NIV, T-2, HT-2, fusarenon-X, diacetoxyscirpenol, neosolaniol, and common modified forms such as DON-3-glucoside or T-2 glucosides where standards exist (Sulyok et al., 2006; Nathanail et al., 2015; Kim et al., 2022; Wu et al., 2023). The weakness is obvious: unknown or unavailable-standard metabolites remain analytically silent unless a broader approach is added.

HRMS addresses that weakness. Orbitrap and QTOF methods can support exact-mass screening, broader suspect lists, retrospective data review, and discovery-oriented analysis of under-characterised analogues or metabolites (Tamura et al., 2015; Rakk et al., 2023; Wu et al., 2023). HRMS is also valuable where the same dataset may later need to be revisited for additional toxins or transformed products not initially targeted. In trichothecene analysis, that broader capability is especially relevant because the metabolite space is larger than the routine commercial standard space.

4.2.6.4 Screening methods and class-oriented approaches

For first-line screening, immunochemical tools remain operationally attractive, especially in food and feed contexts. ELISA and lateral-flow formats can rapidly flag samples for follow-up, but they should be interpreted as class- or subgroup-responsive methods rather than definitive structural assays (Aamot et al., 2013; Oplatowska-Stachowiak et al., 2017). Their evidential value is strongest when used in a tiered framework, not in isolation.

A more structurally informed class-oriented approach has also emerged from mass spectrometry. Because type A and type B trichothecenes share subclass-dependent fragmentation tendencies, precursor-ion and related class-screening strategies can be used to search for unknown trichothecenes on the basis of common fragment behaviour rather than exact prior inclusion on a target list. González-Jartín et al. showed that untargeted MS can detect emerging type A trichothecenes from *Fusarium* cultures by exploiting common fragmentation logic, and Mayer et al. later extended this concept by using machine learning to identify discriminative HRMS fragments that

could drive precursor-ion-scan workflows for type A and type B trichothecenes, including under-characterised analogues (González-Jartín et al., 2018; Mayer et al., 2023). These are not yet routine regulatory methods, but they are important because they point toward forensic-style screening strategies for structurally related toxins in the absence of standards.

For DON-related metabolites, chemistry-driven class expansion is also important. The identification of naturally occurring glutathione, cysteine, cysteinylglycine, and *N*-acetylcysteine conjugates shows that thiol-linked metabolites are not merely synthetic curiosities but real analytes that may function as biomarkers or evidence of transformation pathways (Uhlig et al., 2016). These cannot be handled well by narrow routine screening methods unless they have been specifically anticipated. In this sense, screening in the trichothecene field increasingly means a combination of targeted parent compounds plus HRMS-enabled suspect or transformation-product awareness.

4.2.6.5 Current LC-MS scientific standard

The current scientific standard is a tiered LC-MS strategy in which targeted LC-MS/MS provides primary quantitative confirmation of known analytes, while LC-HRMS provides broader structural scope and retrospective capability.

For routine cereal analysis, triple-quadrupole LC-MS/MS remains the most mature platform. Methods such as those of Sulyok et al., Nathanail et al., Kim et al., and Wu et al. show that multi-analyte targeted determination of both type A and type B trichothecenes is now feasible with acceptable validation performance across wheat, maize, oats, and related materials (Sulyok et al., 2006; Nathanail et al., 2015; Kim et al., 2022; Wu et al., 2023). These methods also illustrate the present compromise: the broader the analyte list, the more care is required with chromatography, ionisation, and matrix compensation.

For structurally broader work, LC-HRMS is increasingly part of the scientific standard rather than an optional add-on. Tamura et al. demonstrated simultaneous determination of 20 *Fusarium* toxins using Orbitrap HRMS with a PFPP column, while Wu et al. showed a one-step pretreatment UHPLC-Q-Orbitrap-HRMS workflow for 16 type A and B trichothecenes and modified forms, supported by isotope-based multipoint calibration (Tamura et al., 2015; Wu et al., 2023). HRMS is especially valuable when the analytical question extends beyond the standard set, for example toward modified toxins, sulfur-linked metabolites, or other transformed analogues.

A key point is that the modern LC-MS standard is not merely measurement of parent toxins. It increasingly includes at least some capacity for modified forms, either by explicit targeted inclusion, by HRMS suspect screening, or both.

4.2.6.6 Functional and orthogonal approaches

Compared with protein toxins or cyanotoxins, trichothecenes have fewer truly distinct orthogonal assay families in routine use, but orthogonality still exists in a useful sense.

First, immunochemical assays provide rapid operational screening. Second, targeted LC-MS/MS provides quantitative structural confirmation of known analytes. Third, HRMS provides broader structural scope and retrospective capability for under-characterised or unavailable-standard

compounds. Fourth, for some specific transformed forms, derivatisation or reaction-based structural clarification may provide further orthogonality. The thiol-conjugation work on DON is a good example: reference reaction mixtures, oxidation to sulfoxides, and product-ion interpretation were used together to establish structure, not just mass match alone (Stanic et al., 2015; Stanic et al., 2016a; Stanic et al., 2016b; Uhlig et al., 2016).

This matters because LC-MS evidence is not always equally strong across all trichothecenes. For parent toxins with standards, retention time, ion ratio, and accurate or nominal mass transitions can be very strong. For novel metabolites, evidence may need to be assembled from exact mass, product ions, chromatographic logic, transformation chemistry, and biological plausibility.

4.2.6.7 Implications for EMBRACE

For EMBRACE, the literature supports a matrix-aware and tiered trichothecene workflow rather than a single universal procedure.

First, for routine structured analysis of known analytes in powders, grains, and similar matrices, a targeted LC-MS/MS method should remain the backbone. It should at minimum include the key type A and type B toxins relevant to the intended threat or occurrence space and, where possible, selected modified forms such as DON-3-glucoside, acetyl-DONs, deepoxy-DON, and major T-2 metabolites.

Second, because the chemistry and current literature make clear that modified forms matter, HRMS capability should be built in as an escalation route or parallel broader-screening route. This is particularly important for evidential, forensic, or investigative applications where absence of a standard should not automatically equal analytical blindness.

Third, the class chemistry argues against overly selective cleanup when the aim includes metabolite discovery or broad suspect screening. Where EMBRACE needs broad structural scope, simpler extraction plus HRMS may be more informative than heavily selective cleanup coupled only to targeted analysis.

Overall, the most defensible EMBRACE position is that trichothecene analysis should be organised as an orthogonal LC-MS-centred workflow: rapid screening where useful, targeted LC-MS/MS for known analytes, LC-HRMS for modified and under-characterised forms, and matrix-specific sample preparation chosen according to whether the priority is robustness, lowest LOQ, or breadth of structural coverage.

4.2.7 Cyclic peptide cyanotoxins: analytical methodology

4.2.7.1 Identity, structure, occurrence, and analytical significance

Microcystins (MCs) are a large family of cyclic heptapeptide cyanotoxins, while nodularin (NOD) is a closely related cyclic pentapeptide produced chiefly by *Nodularia spumigena*. Their analytical and toxicological importance is closely tied to a shared unusual β-amino acid side chain, Adda — (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid — which is a defining structural feature of both microcystins and nodularin and is central to many immunochemical,

chemical, and total-toxin analytical strategies (Namikoshi et al., 1992; Kaya & Sano, 1999; Fischer et al., 2001). Adda is also a major determinant of biological activity, and methods that target the Adda moiety, such as Adda-directed ELISAs or oxidative conversion to MMPB, therefore exploit a chemically conserved motif rather than a single individual congener (Kaya & Sano, 1999; Fischer et al., 2001).

The canonical microcystin structure is usually written as cyclo-(-D-Ala^{1}-X^{2}-D-MeAsp^{3}-Z^{4}-Adda^{5}-D-Glu^{6}-Mdha^{7}), where the amino acids at positions 2 and 4 vary and are commonly used to name the congener. Thus, in MC-LR, leucine occupies position 2 and arginine position 4; in MC-RR, both variable positions are arginine; and so forth (Namikoshi et al., 1992; Luukkainen et al., 1993). Nodularin is structurally simpler but analogous in analytical terms because it also contains the Adda unit and shares similar toxicological and detection behaviour in many assays (Fischer et al., 2001). Beyond the variable X and Z residues, further structural diversity arises from demethylation, dehydroamino-acid variation, and related substitutions at other positions, including changes affecting D-MeAsp, Mdha/Dha/Dhb-type residues, or the Adda-associated framework itself. This produces a very large congener space, much larger than the set of commercially available standards (Fastner et al., 1999; Birbeck et al., 2019; Bouaïcha et al., 2019).

This structural diversity is analytically significant for several reasons. First, congeners differ in polarity, ionisation efficiency, fragmentation behaviour, and chromatographic retention, which affects both targeted LC–MS/MS performance and the reliability of immunochemical screening. Second, the conserved Adda moiety makes broad class-level detection possible, but this same feature means that Adda-based assays can report total or near-total class response without resolving individual congeners. Third, some congeners are isobaric or near-isobaric and may require orthogonal evidence, such as retention behaviour, derivatisation, or high-resolution fragmentation, for confident identification (Miles et al., 2012; Miles et al., 2013; Birbeck et al., 2019). The analytical problem is therefore not simply detection of "microcystin" as a single toxin, but characterization of a structurally diverse toxin family in which conserved and variable motifs must both be taken into account.

From an occurrence perspective, microcystins are primarily associated with freshwater cyanobacterial blooms, especially from genera such as *Microcystis*, *Planktothrix*, *Dolichospermum/Anabaena*, and *Nostoc*, whereas nodularin is classically linked to brackish systems dominated by *Nodularia spumigena* (Luukkainen et al., 1993; Fastner et al., 1999). In real samples, these toxins may occur as intracellular toxins within cyanobacterial biomass, as dissolved extracellular toxins in water after cell lysis, and in exposed organisms as extractable free toxins or covalently protein-bound forms. That distribution across forms and matrices is one of the main reasons why sample preparation is as critical as detector choice in current analytical practice (Williams et al., 1997; Neffling et al., 2010; Cadel-Six et al., 2014).

4.2.7.2 Sample preparation in current practice

For microcystins and nodularin, current analytical practice is driven by matrix diversity as much as by detector capability. Laboratories may need to address relatively clean water, particulate-rich environmental suspensions, wipe and swab extracts, beverages, complex foods, bloom materials, and biological samples such as serum, urine, liver, fish, shellfish, or vomitus. Across these matrices, the main analytical questions are not identical: whether the aim is dissolved toxin, intracellular toxin, freely

extractable toxin, or total toxin including covalently bound forms will determine the sample preparation strategy (Lawton et al., 1994; Turner et al., 2018; Bouteiller et al., 2022).

For water and environmental samples, a key early distinction is whether dissolved toxin alone or combined free plus cell-associated toxin is to be measured. Whole-water approaches commonly use freeze–thaw cycling and/or sonication to lyse cyanobacterial cells before filtration or direct analysis, whereas dissolved-phase methods remove biomass first and analyse the supernatant or filtrate (Rapala et al., 2002; Roy-Lachapelle et al., 2019). For relatively clean waters, direct injection LC–MS/MS or on-line SPE LC–MS methods can be effective, especially when high sensitivity is required without lengthy offline preparation (Beltrán et al., 2012; Fayad et al., 2015; Ortiz et al., 2017; Roy-Lachapelle et al., 2019). For more dilute samples or broader congener coverage, SPE remains standard, most often with C18, HLB, SDB-type, or related sorbents, and careful control of washing conditions is needed to balance desalting and analyte recovery, especially for more polar congeners (Lawton et al., 1994; Beltrán et al., 2012; Munoz et al., 2017; Roy-Lachapelle et al., 2019).

For cyanobacterial bloom material and cultured biomass, solvent extraction with aqueous methanol is still the dominant approach, with 70–90% methanol commonly used, sometimes acidified, often after freeze–thaw disruption or sonication (Fastner et al., 1998; Turner et al., 2018). These extracts may be analysed directly by LC–MS/MS if sufficiently clean, but cleanup or dilution is often helpful when biomass is dense or matrix burden is high. For forensic or incident-response purposes, bloom slurries and scums should be treated as especially heterogeneous, so homogenisation and subsampling control become important pre-analytical steps rather than trivial details.

For wipe and swab samples, the literature is much less mature than for waters and blooms, but the chemistry suggests that recovery-oriented workflows should favour aqueous-organic extraction, typically methanol/water or acetonitrile/water mixtures, with acidic conditions only when compatible with downstream analysis. Because surface wipes may contain dust, soils, humic matter, disinfectant residues, or packaging contaminants, cleanup by SPE or simple dilution may be necessary before LC–MS. The practical challenge is not likely to be intrinsic detectability of microcystins by MS, but uncertain recovery from the sampling substrate and strong surface-derived matrix effects. In that setting, matrix-spiked wipe recovery studies and procedural surrogates become important if the workflow is to support evidential interpretation rather than only qualitative screening.

For beverages and foodstuffs, sample preparation becomes strongly matrix-dependent. Clear waters are analytically easy; juices, plant-derived drinks, and nutrient-rich beverages are not. Fish, shellfish, and algal supplement powders have been successfully addressed by methanolic extraction, often around 75–80% methanol, with subsequent dilution, filtration, and sometimes SPE cleanup (Geis-Asteggiante et al., 2011; Turner et al., 2018). Turner et al. showed that a relatively simple UHPLC–MS/MS workflow could be extended across natural waters, cyanobacteria, shellfish, and algal supplement tablet powders, which is particularly relevant to high-throughput regulatory and surveillance contexts (Turner et al., 2018). For shellfish and fish tissues, QuEChERS-type approaches or PRiME/pass-through cleanup have also been explored, but matrix effects remain non-trivial and method performance can vary substantially by tissue type and congener panel (Geis-Asteggiante et al., 2011; Xu et al., 2019).

For biofluids and tissues, several targeted workflows exist, showing that microcystins can be detected in biological matrices. Human urine has been analysed by immunocapture LC–MS/MS for MC-LR and related targets, and immunocapture-PP2A approaches have also been described for activity-based detection in urine (Wharton et al., 2018; Wharton et al., 2019). Serum and plasma have been analysed by LC–MS/MS and ELISA-based methods, though matrix effects and congener-dependent recovery remain important limitations (Heussner et al., 2014; Palagama et al., 2018). In tissues such as liver, fish, shellfish, and bird or mammalian post-mortem specimens, conventional extraction recovers only the free fraction; total-form analysis requires additional chemistry, discussed below (Neffling et al., 2010; Foss et al., 2018; Bouteiller et al., 2022).

Overall, the main lesson from the sample-preparation literature is that microcystin/nodularin analysis is not one method but a family of workflows shaped by matrix, target fraction, and decision context. A routine water-screening method, a bloom-characterization workflow, a shellfish monitoring method, and a biofluid confirmation method may all use LC–MS, yet require substantially different front-end treatment.

4.2.7.3 Analytical methods used: overview

The most widely used analytical families for microcystins and nodularin are immunochemical assays, protein phosphatase inhibition assays, and chromatographic methods, especially LC–MS/MS and increasingly HRMS. Historically, HPLC-UV and related approaches were important, but their limited selectivity and sensitivity have made them less attractive for modern confirmatory work except in specific settings (Lawton et al., 1994; Rapala et al., 2002).

ELISA remains one of the most practical broad screening tools because Adda-directed antibodies permit class-responsive detection of many congeners at low concentrations with relatively simple equipment and high throughput (Fischer et al., 2001; Heussner et al., 2014). However, ELISA does not resolve congener profiles and may over- or under-respond depending on the exact analyte mixture and the nature of any degradation or transformation products present (Guo et al., 2017; Birbeck et al., 2019). That makes ELISA valuable for screening and triage, but weaker as a stand-alone confirmatory or attribution method.

Protein phosphatase inhibition assays measure biological activity rather than structure. They are attractive because they respond to toxic potency at the protein phosphatase target level, but by the same token they are not structurally specific and can be influenced by other inhibitors or matrix components unless sufficient cleanup is applied (An & Carmichael, 1994; Rapala et al., 2002; Moore et al., 2016). In practice, they are best viewed as functional screening tools complementary to structural analysis.

LC–MS/MS is the current mainstay for specific confirmatory analysis of free microcystin congeners and nodularin. Triple-quadrupole SRM/MRM methods are widely used for targeted quantitation when standards are available and are now well established for waters, bloom extracts, shellfish, fish, and supplement materials (Mekebri et al., 2009; Kaloudis et al., 2013; Turner et al., 2018). The principal weakness is obvious: targeted quantitation is restricted by the standard set and transition list. Since

only a small fraction of known congeners are commercially available, targeted LC–MS/MS can underestimate diversity in real samples (Birbeck et al., 2019).

HRMS addresses part of that limitation by enabling exact-mass screening, retrospective data review, and suspect or non-target interrogation. QTOF and Orbitrap platforms have been used both for broader targeted screening and for identification of less common or previously unrecognized variants, including isobaric or modified congeners that would be difficult to resolve by standard triple-quadrupole methods alone (Ortiz et al., 2017; Roy-Lachapelle et al., 2019; Birbeck et al., 2019). HRMS also becomes especially useful when sample availability is limited and analysts want the same dataset to support both routine targeted reporting and later re-interrogation.

4.2.7.4 Screening methods and total-toxin approaches

A practical analytical framework for microcystins and nodularin usually separates rapid screening of free toxins from more specialised approaches for total toxin content.

For first-line screening, Adda-ELISA and PP2A/PPIA remain the main class-level tools. Adda-ELISA is especially useful when the purpose is to establish whether a sample contains material consistent with microcystins or nodularin at levels relevant to decision-making, while PP2A assays are useful when a functional toxicity-oriented signal is desired (Fischer et al., 2001; Rapala et al., 2002; Guo et al., 2017). These assays are operationally valuable for water surveillance, bloom triage, and high-volume monitoring, but they do not solve the congener-specific confirmation problem.

For total toxin analysis, the reference approach in the literature remains the MMPB/Lemieux oxidation strategy, in which the conserved Adda moiety is oxidatively cleaved to produce MMPB, allowing estimation of total microcystin/nodularin content including bound forms (Kaya & Sano, 1999; Neffling et al., 2010; Munoz et al., 2017; Bouteiller et al., 2022). This is particularly important in tissues and biological matrices, where a substantial fraction may be covalently associated with proteins and therefore invisible to ordinary solvent extraction. The MMPB approach is powerful precisely because it measures a class-level conserved fragment, but this is also its main drawback: it sacrifices congener identity. It is therefore best suited to total-burden questions rather than detailed structural characterization.

Alternative total-form approaches exist, including base-catalysed deconjugation of thiol-conjugated or bound forms, ozonolysis, and more experimental strategies such as laser irradiation desorption, but these remain less established than MMPB and are not yet the routine standard across matrices (Miles et al., 2016; Miles, 2017; Bouteiller et al., 2022). Among these, base-catalysed deconjugation is especially interesting because it may preserve more congener-level information than oxidative total-toxin approaches, but the evidence base is still much thinner and matrix-specific validation is limited (Miles et al., 2016; Foss et al., 2018; Bouteiller et al., 2022).

4.2.7.5 Current LC–MS scientific standard

The present scientific standard for structurally specific analysis is a tiered LC–MS strategy in which targeted LC–MS/MS remains the primary quantitative platform for known congeners, while HRMS provides breadth, retrospective review, and support for variant discovery.

For high-throughput regulatory and monitoring use, rapid UHPLC–MS/MS methods with simple methanolic extraction and minimal cleanup have proven feasible across multiple matrices. Turner et al. developed and single-laboratory validated a method covering natural waters, cyanobacteria, shellfish, and algal supplement tablet powders, showing that a common analytical backbone can be adapted across matrix types when the target is the free toxin fraction (Turner et al., 2018). That is particularly relevant for EMBRACE because it suggests a practical route toward harmonised cross-matrix workflows rather than entirely separate chemistry for every material class.

For broader environmental surveillance and wider cyanotoxin coverage, on-line SPE coupled to HRMS offers clear advantages. Roy-Lachapelle et al. demonstrated a rapid multiclass method for lake waters combining on-line SPE, UHPLC, and Orbitrap HRMS, with low-ng/L detection and the ability to conduct retrospective screening beyond the initial target list (Roy-Lachapelle et al., 2019). Ortiz et al. likewise showed the value of on-line concentration plus QTOF-HRMS for targeted and non-targeted cyanotoxin work, including historical data review (Ortiz et al., 2017). These studies matter because they show that HRMS is not only a discovery tool but also an operational platform for surveillance-grade work.

Most importantly for structural ambiguity, HRMS combined with chemical derivatization has become one of the key routes for resolving problematic congeners. Birbeck et al. used on-line concentration LC–MS/MS, HRMS, and thiol derivatization to identify Dhb-containing microcystins and to demonstrate that nominally targeted assignments could in fact conceal isomeric or mischaracterized congeners (Birbeck et al., 2019). That work is especially instructive because it shows that chromatographic retention, qualifier ratios, exact mass, and derivatization behaviour all contribute useful evidence. In other words, the current LC–MS scientific standard is not merely "run an MRM method," but rather integrate chromatography, accurate mass, fragmentation, and, when needed, structure-probing chemistry.

4.2.7.6 Functional and orthogonal approaches

Even though LC–MS is the structural anchor, orthogonal approaches remain important. For microcystins and nodularin, the most useful orthogonal categories are:

- immunochemical screening, especially Adda-ELISA, for rapid class-responsive detection;
- functional assays, especially PP2A/PPIA, for phosphatase-inhibition evidence;
- total-toxin chemistry, especially MMPB oxidation, for free plus bound burden;
- structural LC–MS confirmation, whether targeted LC–MS/MS or HRMS.

This combination is analytically stronger than any single method alone. Adda-ELISA may flag broad class presence; PP2A may indicate biologically relevant inhibition; LC–MS/MS may quantify the known free congeners; and MMPB may reveal that the actual total burden is higher because of bound material. In richer matrices such as tissues or complex foods, that orthogonality is especially valuable because disagreement between methods can itself be informative. For example, higher ELISA or total-toxin responses than free-congener LC–MS signals may suggest either unmonitored congeners, transformed products, or bound fractions requiring further workup (Foss & Aubel, 2015; Foss et al., 2018; Bouteiller et al., 2022).

4.2.7.7 Implications for EMBRACE

For EMBRACE, the literature points toward a tiered and matrix-aware analytical strategy rather than a single universal method.

First, because matrices may include environmental waters, wipe and swab samples, beverages, foodstuffs, and biofluids, method development should begin by grouping matrices according to extraction behaviour rather than according to administrative sample categories. At minimum, one can distinguish aqueous environmental samples, particulate-rich or biomass-rich samples, organic-rich food or beverage matrices, and biological matrices including tissues and fluids.

Second, for the free toxin fraction, a practical EMBRACE baseline would be a targeted LC–MS/MS method covering the main available congeners, supported where possible by HRMS capability for suspect screening and retrospective review. The studies of Turner et al. and Roy-Lachapelle et al. are particularly relevant here because they show that relatively streamlined extraction and fast chromatography can still deliver useful performance across multiple matrices or multiple cyanotoxin classes (Turner et al., 2018; Roy-Lachapelle et al., 2019).

Third, because the literature clearly shows that free toxin analysis can underestimate total burden, especially in tissues and some exposure contexts, EMBRACE should consider whether a total-toxin option is needed for selected matrices. At present, MMPB remains the most established route for that purpose, although it should be framed explicitly as a class-total method rather than a congener-resolved one (Neffling et al., 2010; Munoz et al., 2017; Bouteiller et al., 2022).

Fourth, where unusual profiles or attribution-relevant questions arise, HRMS plus derivatization offers a realistic escalation path. Birbeck et al. demonstrate why that matters: apparent targeted identifications may not be chemically sufficient when isomeric or Dhb/Mdha-related ambiguities exist (Birbeck et al., 2019).

Overall, the most defensible EMBRACE position is that microcystin/nodularin analysis should be organised as an orthogonal workflow: rapid class-level screening where needed, targeted LC–MS/MS for known congeners, HRMS for broader structural scope, and total-toxin chemistry for selected matrices where bound forms may be important.

4.3 Method development status

4.3.1 Integrated analytical strategy and current development concept

4.3.1.1 Strategic objective

The objective of the EMBRACE analytical strategy is to enable broad and uniform laboratory screening of unknown samples for the full project toxin panel without requiring correct toxin-class assignment at the point of sample receipt. This is necessary because incident-related samples may arrive with only limited prior information, uncertain matrix composition, and no reliable indication of whether the relevant toxic agent is a low-molecular-weight compound, a protein toxin, or a mixture of both. The

strategy therefore aims to maximise practical toxin coverage at the initial analytical stage while retaining a clear route to higher-specificity follow-up and confirmation.

4.3.1.2 Operational concept

The current EMBRACE concept is based on a single integrated intake-and-screening pathway for unknown samples. All samples enter a common reception and routing framework, are subjected to initial matrix assessment, and are assigned to a limited number of broad operational intake classes on the basis of declared sample type, case information, physical form, and apparent matrix characteristics. This intake step is not intended to fully determine sample composition, but to support practical routing into a workflow that preserves broad analytical coverage.

Following intake, samples are directed into parallel analytical branches for low-molecular-weight toxins and protein toxins. The low-molecular-weight branch is based on LC–MS screening using complementary hydrophilic interaction and reversed-phase chromatographic channels, while the protein-toxin branch combines immunological screening, selective enrichment, functional assays, and peptide-level mass spectrometric confirmation. Positive or ambiguous findings are then escalated to toxin-class-appropriate orthogonal confirmation. In this way, the strategy maintains a single unified entry point and decision framework while accommodating the different chemical and biochemical requirements of the toxin classes involved.

4.3.1.3 Analytical design principles

The EMBRACE strategy is based on four main analytical design principles.

First, all unknown samples are handled within one common analytical framework rather than through separate toxin-specific entry workflows. This reduces the risk that relevant toxin classes would be excluded prematurely on the basis of incorrect initial assumptions.

Second, samples are assigned only to broad operational matrix classes rather than narrowly predefined sample categories. This reflects the practical reality that full sample composition is not usually known at intake and that analytical routing must therefore rely on limited but actionable information.

Third, broad screening precedes analytical specialisation. For low-molecular-weight toxins, this means prioritising a harmonised LC–MS-centred screening concept with broad target and suspect coverage. For protein toxins, it means applying a tiered branch in which screening, functional evidence, and structural peptide confirmation contribute complementary information. In both cases, the initial aim is broad toxin-class coverage rather than immediate analyte-specific optimisation.

Fourth, definitive identification is based on orthogonal confirmation. Screening results alone are not considered sufficient for high-confidence assignment where evidential demands are greater. Instead, structurally, functionally, or immunologically independent confirmatory evidence is introduced according to the toxin class detected and the analytical question being addressed.

4.3.1.4 Current development status

The work carried out to date has established the overall direction of this strategy and has begun to translate it into draft operational workflows. The low-molecular-weight toxin branch is currently the

most harmonised part of the system. Development work has supported convergence toward a common sample-preparation concept, limited matrix grouping, two complementary LC channels, and an HRMS-first screening model with triple-quadrupole LC–MS/MS as targeted follow-up. This branch therefore already provides the clearest basis for a unified cross-class workflow.

For protein toxins, the development status is more modular but the strategic logic is also established. Current draft and partially developed modules include sandwich ELISA-based screening for ricin and BoNT/A, Endopep-based functional detection of active botulinum neurotoxins, activity-linked ricin detection through adenine-release LC–MS/MS, and targeted peptide-level LC–MS/MS confirmation for both ricin and BoNT. Alternative enrichment approaches, including lectin-based ricin capture, remain relevant in suitable matrices. Although these protein-toxin elements are not yet harmonised to the same degree as the low-molecular-weight LC–MS workflow, together they already define a coherent protein-toxin branch within the overall EMBRACE pathway.

Overall, the current status can therefore be described as strategically unified but analytically modular. The integrated EMBRACE pathway is now defined at concept level, its main screening and confirmation elements have been identified, and several of these have already been drafted into preliminary operating procedures. The remaining work is primarily to refine, optimise, and evaluate the selected modules across representative matrices and to translate the present development framework into robust EMBRACE-relevant operating procedures.

4.3.2 Sample intake, matrix grouping, and routing logic

4.3.2.1 Purpose of the intake step

The intake step is intended to support practical analytical routing of unknown samples while preserving broad toxin coverage. It is not intended to provide full compositional characterisation of the sample at the point of receipt. In an incident-related context, samples may arrive with only limited information regarding origin, composition, or likely toxin class. The EMBRACE workflow therefore uses a limited operational assessment based on the information available at receipt rather than requiring detailed prior knowledge of the sample.

4.3.2.2 Initial sample assessment

At receipt, each sample is assessed on the basis of declared sample type, case context, physical form, visible matrix characteristics, and available sample amount. The aim of this assessment is to determine how the sample should enter the integrated EMBRACE pathway, not to assign a definitive toxin class. In practical terms, this means identifying whether the sample is liquid or solid, whether it appears compositionally simple or rich in proteins, lipids, or particulates, and whether sufficient material is available for parallel processing.

4.3.2.3 Operational matrix grouping

To keep the workflow manageable, samples are assigned to a limited number of broad operational intake classes rather than narrowly defined matrix categories. At minimum, these classes distinguish between liquid and solid samples and between relatively non-rich and matrix-rich materials. Thus, waters, dilute beverages, and similar samples may be handled as liquid non-rich matrices, whereas

serum, milk, and other protein- or lipid-rich liquids are treated as liquid rich matrices. Powders, soils, sediments, dry foods, and homogenised materials enter as solid matrices, with additional matrix-relief steps introduced only where needed. This broad grouping approach is intended to support robust routing across diverse sample types without requiring detailed compositional analysis before screening begins.

4.3.2.4 Common routing principle

Following intake classification, all samples enter a common EMBRACE routing framework. Where sample amount permits, aliquots are prepared for parallel progression into both the low-molecular-weight toxin branch and the protein-toxin branch. This parallel-routing principle is a central feature of the strategy. Because unknown samples cannot reliably be assumed to contain only one toxin class, the default approach is to preserve broad analytical coverage rather than to exclude one branch prematurely.

4.3.2.5 Routing to the low-molecular-weight toxin branch

Routing into the low-molecular-weight toxin branch is governed primarily by analyte polarity and chromatographic compatibility. After common front-end handling, the workflow is designed to generate fractions suitable for two complementary LC–MS channels: a hydrophilic channel for highly polar analytes such as paralytic shellfish toxins, and a reversed-phase channel for less polar targets including microcystins and nodularin, trichothecenes, aconitum alkaloids, strychnine-type alkaloids, and related compounds. The purpose of this branch is therefore not to create separate workflows for each toxin class, but to accommodate the major low-molecular-weight toxin groups within one harmonised LC–MS-centred analytical system.

4.3.2.6 Routing to the protein-toxin branch

Routing into the protein-toxin branch is governed primarily by the type of evidence required rather than by analyte polarity. Samples entering this branch may undergo immunological screening, selective enrichment, functional testing, and peptide-level mass-spectrometric confirmation as required. Matrix complexity and available sample amount are especially important in this branch, because they influence whether direct immunological screening, affinity-based enrichment, functional assays, or peptide-level confirmation are most appropriate. Even so, the branch remains part of the same overall EMBRACE pathway and is not treated as a separate standalone workflow.

4.3.2.7 Handling of limited or constrained samples

The workflow is designed for flexibility where sample amount is limited. If material is insufficient for full parallel processing, routing may prioritise the analytically most informative branch on the basis of matrix type, case context, and expected evidential value, while retaining reserve material for secondary follow-up where possible. This does not alter the overall strategy, but recognises that broad parallel screening may sometimes need to be adapted to practical sample constraints.

4.3.2.8 Role of the intake decision

An important feature of this routing concept is that the intake decision is deliberately conservative. Its purpose is to support broad screening, not to determine the final analytical identity of the sample.

Analytical specialisation is introduced progressively after screening, not at the point of receipt. This reduces the risk that relevant toxin classes would be missed because of incorrect up-front assumptions and is consistent with the broader EMBRACE principle that unknown samples should first be handled through a common, coverage-oriented pathway and only later directed to more specialised confirmatory analysis.

4.3.2.9 Current outcome of the routing concept

The routing logic developed in Task 3.2 therefore establishes the operational backbone of the EMBRACE analytical strategy. A single intake concept is maintained for all samples, matrix classification is kept deliberately broad, and parallel analytical branches are used to preserve coverage across both low-molecular-weight and protein toxins. This provides a practical balance between analytical breadth and operational manageability and forms the basis for the branch-specific workflows described in the following sections.

4.3.3 Low-molecular-weight toxin screening branch

4.3.3.1 Purpose of the low-molecular-weight branch

The purpose of the low-molecular-weight toxin branch is to provide broad and operationally uniform screening of the EMBRACE low-molecular-weight toxin panel within the integrated analytical pathway described above. This branch is intended to cover the principal small-molecule toxin classes of interest without requiring a separate end-to-end workflow for each class. The development objective has therefore been to establish a harmonised LC–MS-centred system that can accommodate chemically diverse analytes through a limited number of shared preparation and separation steps.

4.3.3.2 General analytical concept

The current concept is based on a common front-end workflow followed by two complementary chromatographic-MS channels. The common front-end is intended to reduce the number of fully separate sample-preparation procedures while still allowing differential routing of analytes according to polarity and matrix compatibility. The two analytical channels are a hydrophilic interaction LC–MS channel for highly polar analytes and a reversed-phase LC–MS channel for less polar analytes. Together, these channels provide broad coverage across the main toxin classes currently prioritised in Task 3.2.

Within this framework, high-resolution LC–MS is used as the principal screening platform. This choice reflects the need for broad target and suspect coverage, accurate-mass precursor information, high-resolution product-ion data, and the possibility of retrospective data review. Triple-quadrupole LC–MS/MS is used as a targeted follow-up layer for predefined analytes where additional sensitivity, faster routine review, or transition-based confirmation is required.

4.3.3.3 Scope of analyte coverage

The branch is intended to cover, at minimum, the low-molecular-weight toxin classes reviewed in Section 4.2. These include paralytic shellfish toxins, microcystins and nodularin, trichothecenes, and plant alkaloid toxins such as aconitum alkaloids and strychnine-type alkaloids. The branch also provides a framework into which additional predefined small-molecule toxins can be incorporated,

provided that they are compatible with one of the two chromatographic channels and with the general sample-preparation logic.

This class-based coverage is one of the main reasons why a harmonised branch was preferred over a set of toxin-specific workflows. The development work showed that, although the analytes differ substantially in polarity and ionisation behaviour, they can still be grouped in a practical way around a limited number of LC–MS conditions rather than requiring completely separate analytical pipelines for each toxin family.

4.3.3.4 Common sample-preparation concept

Method development has supported a common preparation concept in which samples, after initial intake classification and any necessary matrix-relief step, are brought into a shared extraction and partitioning logic. For solids, this generally involves extraction into an aqueous-organic intake extract. For liquids, the sample may enter the workflow more directly unless additional protein or lipid reduction is required. Where needed, a short protein-precipitation or matrix-relief step is used for rich matrices such as serum, plasma, or milk.

After this initial handling, the current workflow concept uses a brief partitioning step to generate analytically useful fractions for downstream routing. In practical terms, this yields an organic branch enriched in analytes suitable for reversed-phase analysis and an aqueous branch retaining analytes better suited to hydrophilic handling. This common split concept is central to the current EMBRACE thinking, because it allows several toxin classes to be covered through one harmonised front-end rather than multiple unrelated extraction procedures.

4.3.3.5 Hydrophilic LC–MS channel

The hydrophilic channel is intended primarily for paralytic shellfish toxins and other highly polar analytes of comparable chromatographic behaviour. Development work and literature review both supported the conclusion that these analytes are poorly suited to conventional reversed-phase LC–MS and are better handled through HILIC-based separation. In the current EMBRACE concept, the aqueous fraction generated during the common preparation workflow is therefore directed, where appropriate, to a dedicated hydrophilic clean-up and HILIC-LC–MS analysis.

This hydrophilic channel represents a key design result of the development work. Rather than forcing all low-molecular-weight toxins into one reversed-phase platform, the strategy explicitly incorporates a dedicated route for the highly polar toxin group. This improves chemical coverage while preserving the overall logic of a single harmonised branch.

4.3.3.6 Reversed-phase LC–MS channel

The reversed-phase channel is intended for less polar or moderately polar low-molecular-weight toxins. In the present EMBRACE strategy, this includes microcystins and nodularin, trichothecenes, aconitum alkaloids, strychnine-type alkaloids, and related compounds. These analytes are sufficiently compatible with RP-LC–MS to be handled within one broader reversed-phase screening branch, even though analyte-specific optimisation may still be needed at the level of gradient design, ion selection, and transition choice.

The RP channel is therefore the principal high-coverage lane for the more MS-compatible low-molecular-weight toxins. It is also the channel into which most of the current cross-class analyte expansion has been built. This makes it an especially important part of the present workflow architecture.

4.3.3.7 HRMS-first screening and targeted QqQ follow-up

A central development decision in this branch has been the positioning of LC–HRMS as the primary screening platform and triple-quadrupole LC–MS/MS as the targeted follow-up platform. This decision reflects the different strengths of the two instrument types. HRMS supports broad inclusion-list and suspect screening, exact-mass review, and recovery of richer structural information from a single acquisition. QqQ methods remain advantageous where analytes are predefined and where additional sensitivity, transition-based selectivity, or faster routine follow-up is needed.

The branch is therefore not based on a choice between HRMS and QqQ, but on a staged use of both. HRMS provides the broad first-pass screening environment, whereas QqQ supports targeted review and confirmation of selected analytes once they have been prioritised. This division of labour is one of the clearest methodological outcomes of the work so far.

4.3.3.8 Current development outputs

The low-molecular-weight branch is currently the most harmonised and most advanced part of the overall EMBRACE analytical strategy. Development work has already produced a draft Recommended Operating Procedure for qualitative LC–MS screening of selected low-molecular-weight toxins. This draft defines the basic matrix-intake logic, the shared preparation concept, the two-channel chromatographic design, the role of surrogate markers, the division between HRMS screening and QqQ follow-up, and preliminary qualitative identification criteria.

In parallel, analyte tables, proposed precursor ions, candidate transitions, and structure-related diagnostic fragments have been assembled for the principal toxin groups. This includes hydrophilic analytes intended for HILIC-LC–MS and reversed-phase analytes intended for RP-LC–MS. Although further optimisation is still required, these assets demonstrate that the branch has progressed beyond general concept formation and into structured method drafting.

4.3.3.9 Current limitations and maturity

Despite this progress, the branch should still be regarded as under development rather than fully mature. The overall architecture is now clear, but several analyte- and matrix-specific elements remain provisional. These include optimisation of selected transitions, retention-time assignment across representative matrices, more systematic evaluation of matrix effects and recoveries, and fuller testing of the routing logic under realistic sample constraints. In addition, some analyte classes are currently better represented than others in the draft tables and working workflows.

Even so, the present state of development is sufficient to support a clear strategic conclusion. The low-molecular-weight toxin problem in EMBRACE is no longer being approached as a set of separate toxin-specific workflows. Instead, it has converged toward one harmonised screening branch with a common intake concept, a shared sample-preparation logic, two complementary chromatographic

channels, and a staged HRMS/QqQ analytical design. This branch therefore provides the strongest current foundation for a unified cross-class screening workflow within Task 3.2.

4.3.4 Protein-toxin screening and confirmation branch

4.3.4.1 Purpose of the protein-toxin branch

The purpose of the protein-toxin branch is to provide broad screening and decision-grade follow-up for protein toxins within the integrated EMBRACE analytical pathway. In contrast to the low-molecular-weight toxin branch, this part of the workflow cannot be reduced to a single harmonised chromatographic solution, because the analytical questions are inherently multi-dimensional. For protein toxins, the laboratory may need to determine not only whether toxin-related material is present, but also whether the toxin is biologically active and whether the detected material can be confirmed at molecular level with sufficient specificity. The development objective has therefore been to establish a tiered branch in which screening, functional evidence, and peptide-level structural confirmation can be combined within one coherent strategy.

4.3.4.2 General analytical concept

The current EMBRACE concept for protein toxins is based on a modular but integrated sequence of analytical layers. The first layer is immunological screening, which provides rapid and sensitive indication of toxin-related material in suitable matrices. The second layer is selective enrichment, which improves compatibility with downstream functional and mass-spectrometric analysis. The third layer is functional detection, which demonstrates catalytically active toxin where such evidence is relevant and analytically feasible. The fourth layer is peptide-level mass spectrometric confirmation, which provides structurally specific evidence for toxin identity.

This branch is therefore not built around a single universal assay, but around a defined sequence of complementary modules. The intention is that these modules should be selected and combined according to matrix type, sample amount, and evidential need, while still remaining part of one common EMBRACE protein-toxin strategy.

4.3.4.3 Current toxin scope

At the present stage, this branch has been developed primarily around ricin and botulinum neurotoxins, which together represent two analytically distinct but operationally relevant protein-toxin models. Ricin development has included immunological screening, functional detection of catalytic activity, and peptide-level mass spectrometric confirmation. BoNT development has similarly included immunological screening, functional Endopep-type assays, and peptide-level mass spectrometric confirmation. These two toxin systems therefore provide the current foundation for the EMBRACE protein-toxin branch and illustrate how the tiered concept can be applied in practice.

4.3.4.4 Immunological screening layer

A main outcome of the development work has been the establishment of immunological screening as the primary entry point for the protein-toxin branch. Draft sandwich ELISA workflows have been assembled for ricin and BoNT/A, including toxin-specific capture and detection antibodies, irrelevant-capture control formats for identification of nonspecific matrix reactivity, and spike-based control

concepts for revealing matrix masking effects. This is an important development result because it provides a practical and relatively rapid screening layer that can be applied early in the workflow before more resource-intensive confirmation steps are undertaken.

The role of this layer is not to provide definitive protein-toxin identification on its own, but to support triage, early indication, and prioritisation of downstream analytical effort. Within the current EMBRACE concept, immunological screening is therefore treated as a sensitive front-end module that feeds into selective enrichment and orthogonal confirmation where required.

4.3.4.5 Selective enrichment and matrix-dependent front-end handling

The development work has also shown that protein-toxin analysis depends strongly on front-end enrichment and matrix-adapted preparation. For ricin, direct extraction or buffer dilution may be sufficient for ELISA-type screening in some matrices, but peptide-level mass spectrometric confirmation in rich or complex matrices benefits from selective capture. Draft and preliminary workflows currently include immunoaffinity-based enrichment as one developed option, particularly for peptide-level LC–MS/MS confirmation, and lectin-based or galactose-type affinity capture remains relevant as an alternative route in matrices that are less analytically challenging or where sufficient sample amount is available.

For BoNTs, immunoenrichment is even more central, because it supports both functional Endopep analysis and peptide-level confirmation. The current strategy therefore treats selective enrichment not as an isolated preparative detail, but as a core part of the protein-toxin evidential chain. This is one of the main reasons why the protein-toxin branch is more modular than the low-molecular-weight LC–MS branch: the front-end cannot be fully standardised in the same way across all analytes and matrices, but still follows a common strategic logic.

4.3.4.6 Functional confirmation modules

A major part of the current protein-toxin branch is the inclusion of functional assays as explicit confirmation modules.

For botulinum neurotoxins, development has centred on Endopep-type assays in which immunoenriched toxin is incubated with SNARE-derived substrates and the resulting cleavage products are detected through multiple possible readout formats. The current draft strategy includes suspension immunoassay, MALDI-TOF MS, and optional LC-ESI-MS detection modes. This is a significant result because it gives EMBRACE access to evidence of catalytically active toxin rather than only immunoreactivity or structural presence.

For ricin, a functional workflow has been defined in which immunocaptured toxin is incubated with an RNA substrate and toxin-mediated adenine release is quantified by LC–MS/MS. This provides an activity-linked confirmation route that complements immunological screening and peptide-level structural confirmation. The availability of this assay is particularly valuable in contexts where functional integrity of the toxin is analytically relevant.

Together, these two modules show that the current EMBRACE protein-toxin branch has moved beyond simple immunological detection and now includes function-based analytical capability for both main protein-toxin targets.

4.3.4.7 Peptide-level mass spectrometric confirmation

Another important result of the development work is that targeted peptide-level LC–MS/MS confirmation is available for both ricin and BoNT. This considerably strengthens the maturity of the protein-toxin branch. For ricin, immunoaffinity-based enrichment followed by tryptic digestion and targeted SRM/MRM analysis has been evaluated in complex matrices including serum, milk, soil/sand, and cat feces, demonstrating that peptide-level confirmation is not limited to neat or simple laboratory samples. For BoNT, targeted peptide-level mass spectrometric confirmation is likewise part of the current analytical concept and provides the structural layer needed to complement immunological and functional evidence.

This peptide-level MS capability is important strategically because it means that the EMBRACE protein-toxin branch already contains the same basic evidential architecture that was identified in Section 4.2 as the current analytical standard: screening, functional evidence, and molecular confirmation. It also means that the branch is more mature than a purely conceptual toolbox. At present, broader non-targeted protein strategies remain of interest as a future development direction, but targeted peptide confirmation provides the simpler and more defensible current route for operational use.

4.3.4.8 Current development outputs

The present protein-toxin branch is already supported by concrete analytical assets. Draft or partially developed operating procedures have been outlined for sandwich ELISA screening of ricin and BoNT/A, functional Endopep-based BoNT detection, and activity-linked ricin detection by adenine-release LC–MS/MS. In addition, targeted peptide-level LC–MS/MS methods for ricin and BoNT have been established at draft or working level as structural confirmation tools. These outputs show that the branch has progressed beyond general strategic intent and into defined analytical modules with practical workflow value.

At the same time, the branch remains less harmonised than the low-molecular-weight toxin branch. This is not primarily a weakness, but a reflection of the analytical nature of protein toxins. The need to preserve activity, the dependence on enrichment reagents, and the value of multiple independent evidence types make a completely uniform one-assay solution unrealistic. The present EMBRACE approach therefore accepts modularity at method level while maintaining integration at workflow level.

4.3.4.9 Current limitations and maturity

Although the strategic architecture of the protein-toxin branch is now clear, further work is still required before the branch can be regarded as fully mature in operational terms. Matrix-dependent performance must still be evaluated more systematically, reagent-dependent steps require continued optimisation and control, and the balance between rapid screening, functional testing, and peptide-

level confirmation needs to be refined for different sample contexts. In addition, wider extension beyond ricin and the currently prioritised BoNT targets remains a longer-term development question.

Nevertheless, a clear strategic conclusion can already be drawn. The EMBRACE protein-toxin problem is not being addressed through isolated toxin-specific assays, but through one integrated branch built on immunological screening, selective enrichment, functional confirmation, and peptide-level structural confirmation. Ricin and BoNT currently provide the strongest worked examples of this branch, and the draft methods developed around them already establish the main operational logic on which later EMBRACE-relevant operating procedures can be built.

4.3.5 Orthogonal confirmation, supporting analytical assets, and current maturity

4.3.5.1 Role of orthogonal confirmation in the EMBRACE strategy

A central outcome of the method-development work is that the EMBRACE analytical pathway is not based on single-method identification claims. Across both the low-molecular-weight and protein-toxin branches, the current strategy relies on orthogonal confirmation, that is, the use of analytically independent evidence types to support or refine the interpretation of screening results. The precise form of orthogonality differs between toxin classes, but the underlying principle is consistent: screening establishes analytical suspicion, whereas higher-confidence identification requires confirmatory evidence derived from a second analytical dimension.

For low-molecular-weight toxins, orthogonality is achieved primarily through the combination of chromatographic selectivity, high-resolution or targeted tandem mass spectrometric evidence, and, where relevant, alternative chromatographic handling of chemically distinct analyte groups. In practical terms, this means that broad HRMS screening can be followed by targeted QqQ analysis, and that highly polar analytes such as paralytic shellfish toxins are handled through a dedicated HILIC route rather than being forced into the same separation space as less polar analytes. For protein toxins, orthogonality is broader in form and may combine immunological screening, selective enrichment, functional evidence, and peptide-level structural confirmation. This tiered evidential logic is now a defining feature of the EMBRACE development concept.

4.3.5.2 Current supporting analytical assets

The work carried out in Task 3.2 has also produced a set of supporting analytical assets that make the current strategy more concrete and operational than a purely conceptual framework. A major result has been the preparation of draft Recommended Operating Procedures or equivalent draft workflow documents for key parts of the analytical pathway. These include a harmonised draft LC–MS screening workflow for selected low-molecular-weight toxins, draft Endopep-based procedures for functional detection of botulinum neurotoxins, a draft workflow for functional ricin detection by adenine-release LC–MS/MS, and draft sandwich ELISA procedures for ricin and BoNT/A screening. Although these documents remain developmental rather than final, they already define the intended analytical logic,

main control concepts, and key decision points in a form that can support further optimisation and internal harmonisation.

In parallel, the project has generated analytical reference assets that support branch-specific implementation. For the low-molecular-weight branch, these include preliminary analyte tables, proposed precursor ions, candidate product ions and transitions, expected adduct forms, diagnostic fragments, and qualitative identification criteria for both hydrophilic and reversed-phase analytes. For the protein-toxin branch, draft peptide targets, capture strategies, and function-based detection concepts have been assembled for the currently prioritised toxin systems. These materials are important because they provide the bridge between broad strategic design and analyte-level method implementation.

4.3.5.3 Analytical decision support and identification criteria

Another significant development result is that the current strategy is increasingly supported by explicit analytical decision rules rather than only by instrument-specific methods. In the low-molecular-weight branch, presumptive identification is being framed around retention behaviour, accurate mass, expected adduct or charge state, expected product ions, and acceptable blank status, with targeted QqQ follow-up used where higher-confidence transition-based confirmation is required. In the protein-toxin branch, analogous logic is emerging through the combination of toxin-specific immunoreactivity, functional readout thresholds, and peptide-level confirmation criteria. This is important because it means that the EMBRACE workflow is starting to define not only how samples are measured, but also how analytical evidence is to be interpreted.

The development of such decision support is especially important in a broad screening context, where the analytical challenge is not merely to detect signal but to distinguish meaningful toxin-related findings from matrix background, nonspecific reactivity, or incomplete evidence. The draft methods developed so far therefore contribute not only instrument procedures, but also the beginnings of a common EMBRACE interpretation framework.

4.3.5.4 Current maturity across the workflow

Taken together, these supporting assets show that the EMBRACE analytical strategy has progressed beyond the stage of literature-informed option assessment and into structured workflow definition. However, the current maturity remains uneven across different parts of the system.

The low-molecular-weight toxin branch is presently the most mature at the level of workflow harmonisation. It has a defined intake logic, a shared preparation concept, two chromatographic channels, a clear HRMS-first screening philosophy, and a targeted QqQ follow-up concept. The protein-toxin branch is currently more mature at the level of analytical module definition than at the level of full workflow harmonisation. Its individual elements—ELISA screening, selective enrichment, functional toxin detection, and targeted peptide-level confirmation—are already sufficiently defined to demonstrate a coherent branch architecture, but the branch still depends more strongly on toxin-specific reagents, matrix-dependent choices, and modular sequencing of evidence.

This difference in maturity should not be interpreted as a lack of strategic coherence. Rather, it reflects the fact that the two major analyte domains present different constraints on harmonisation. Low-molecular-weight toxins can be grouped more readily around common chromatographic and mass-spectrometric principles, whereas protein toxins require a more explicit combination of immunological, functional, and molecular evidence types. In both cases, however, the method-development work has already produced enough structure to justify the current integrated EMBRACE pathway.

4.3.5.5 Implications for the next development stage

The current state of orthogonal confirmation and supporting assets also clarifies the nature of the remaining work. The main task is no longer to define the broad analytical direction, but to strengthen the existing framework through optimisation, matrix-specific performance assessment, and consolidation of the draft procedures into more stable operating documents. In practical terms, this means refining analyte-specific LC–MS parameters, expanding representative matrix testing, improving control concepts where necessary, and ensuring that the orthogonal confirmation logic is consistently translated into working analytical criteria.

Overall, the present supporting assets already provide a substantial basis for further development. They demonstrate that the EMBRACE strategy is no longer only a conceptual model, but an analytical system with identifiable workflow components, draft procedures, preliminary interpretation rules, and clear lines of orthogonal confirmation across both major branches.

4.3.6 Remaining gaps and next development steps

4.3.6.1 Remaining technical gaps

Although the overall EMBRACE analytical pathway is now defined, a number of technical gaps remain before the selected workflow elements can be regarded as mature operational procedures. In the low-molecular-weight toxin branch, the main remaining needs relate to analyte- and matrix-specific optimisation. These include refinement of selected precursor and product ions, evaluation of chromatographic retention behaviour across representative matrices, further assessment of recoveries and matrix effects, and confirmation that the proposed routing logic remains robust when applied to realistic sample sets rather than idealised developmental cases. The overall two-channel LC–MS architecture is now clear, but the level of optimisation is still uneven across toxin classes and analytes.

In the protein-toxin branch, remaining technical gaps are more strongly associated with enrichment, reagent dependence, and matrix variability. Functional assays and peptide-level confirmation routes have been defined for the main current targets, but their performance remains sensitive to matrix composition, antibody behaviour, capture efficiency, and preservation of toxin activity. Additional work is therefore needed to clarify in which matrix contexts particular enrichment and confirmation combinations are most effective and how robustly these modules can be transferred into a more routine operating format.

4.3.6.2 Validation and performance-evaluation needs

A second major area of remaining work concerns formal performance evaluation. Much of the current development has focused on workflow architecture, analytical logic, and draft implementation. The next stage must place greater emphasis on performance characteristics across representative matrices and toxin classes. This includes evaluation of sensitivity, selectivity, repeatability, matrix tolerance, and practical robustness for the selected workflow elements. It also includes further development of analytical decision thresholds and interpretation criteria so that presumptive screening findings and orthogonally confirmed findings can be handled in a more standardised way across the workflow.

This need applies to both major branches of the EMBRACE strategy. For the low-molecular-weight branch, it concerns the extent to which the harmonised HRMS/QqQ concept performs consistently across different analyte groups and matrix categories. For the protein-toxin branch, it concerns not only assay sensitivity and selectivity, but also the reliability of combining immunological, functional, and peptide-level evidence under different sample conditions.

4.3.6.3 Workflow consolidation needs

In addition to technical optimisation and performance assessment, the strategy still requires further consolidation at the procedural level. Draft Recommended Operating Procedures and related workflow documents already exist for several key components, but these remain developmental documents rather than stable operational procedures. The next phase of work must therefore convert the current draft framework into a more internally harmonised and decision-ready set of EMBRACE-relevant procedures.

In practical terms, this includes consolidation of control concepts, clearer alignment of screening and follow-up rules, refinement of analyte tables and peptide targets, and more consistent translation of branch-specific analytical evidence into common interpretation logic. It also includes clarification of where harmonisation is appropriate and where deliberate modularity must be retained, particularly in the protein-toxin branch.

4.3.6.4 Future development directions

Some additional development directions are also identifiable, but these should be regarded as extensions of the current strategy rather than prerequisites for it. For low-molecular-weight toxins, future work may expand analyte coverage, improve multiclass integration, and strengthen orthogonal options for selected challenging toxin groups. For protein toxins, broader non-targeted or discovery-oriented proteomic approaches remain of interest as longer-term developments, particularly where they might extend subtype coverage or support more open-ended investigation. However, these broader protein-MS strategies are not required to define the current EMBRACE pathway and should be regarded as future enhancements rather than immediate core elements.

4.3.6.5 Overall next steps

The main conclusion is therefore that the remaining work is no longer primarily strategic in nature. The integrated EMBRACE pathway, its two major analytical branches, and its orthogonal confirmation philosophy are now established. The next development stage is instead concerned mainly with optimisation, performance evaluation, and procedural consolidation. This is an important transition point in Task 3.2: the emphasis can now shift from deciding the analytical direction to stabilising the selected workflow elements so that they can support robust and transferable EMBRACE operating procedures.

4.4 Analytical performance considerations

4.4.1 Performance objective in EMBRACE

4.4.1.1 Primary analytical objective

The primary analytical objective in EMBRACE is **defensible qualitative identification** rather than routine quantitative reporting. The workflow is intended to support screening, detection, and identification of biological toxins in unknown samples relevant to incident response, forensic examination, and laboratory verification. The central performance question is therefore whether the analytical pathway can support a justified and reviewable decision on the presence or absence of a toxin under predefined identification criteria.

4.4.1.2 Qualitative rather than quantitative scope

This distinction is important because the EMBRACE strategy covers multiple toxin classes and diverse matrices for which full quantitative method development would not be realistic or necessary within the project scope. EMBRACE is not intended to establish a universally quantitative platform for routine environmental, food, or clinical monitoring. If selected analytical modules were later adapted for such purposes, separate quantitative validation would be required for the relevant analytes and matrices. Within EMBRACE itself, however, the intended analytical use remains qualitative.

4.4.1.3 Why performance characterisation is still required

A qualitative purpose does not remove the need for analytical performance characterisation. Reliable qualitative identification still requires defined sensitivity, selectivity, and robustness. The workflow must therefore be characterised sufficiently to demonstrate that relevant toxins can be detected and identified at low levels under the applicable decision rules. In this context, the purpose of performance assessment is not routine concentration assignment, but defensible qualitative detection and identification.

4.4.1.4 Role of detection limits and sensitivity estimates

Accordingly, limits of detection, detection capability, or equivalent sensitivity estimates are still required for the selected analytes and analytical modules. Where authentic reference standards are available, such estimates can be established directly for the target analyte in the relevant matrix. Where standards are unavailable, sensitivity may need to be approximated using structurally related reference compounds, representative congeners, practical signal thresholds, or evidence-based identification limits derived from the screening workflow. Thus, even in a qualitative framework, the analytical sensitivity of the method must still be defined at least at an estimated level.

4.4.1.5 How performance is interpreted in EMBRACE

Within EMBRACE, analytical performance is understood as the combined outcome of sample preparation, matrix compatibility, screening breadth, confirmatory specificity, and quality-control-supported interpretation. Quantitative capability may still be relevant for selected methods or possible future applications, but it is not the organising principle of the present strategy. Instead, performance

is judged by whether the workflow can support reliable toxin identification across realistic sample types while making uncertainty, orthogonality, and analytical limitations explicit.

4.4.1.6 Matrix- and toxin-class-aware application

A further consequence of this approach is that EMBRACE does not rely on a single generic performance model across all toxin classes. The analytical requirements for highly polar low-molecular-weight toxins, less polar LC–MS-compatible toxins, and protein toxins are not identical. Performance must therefore be assessed in a toxin-class-aware and matrix-aware manner, while still remaining aligned with one common objective: robust and defensible qualitative identification of toxins in unknown samples.

4.4.2 Core analytical performance characteristics

4.4.2.1 Overview

Within the qualitative EMBRACE framework, three analytical performance characteristics are of central importance: **sensitivity**, **specificity/selectivity**, and **robustness**. These are treated as core properties of the overall workflow rather than of the detector alone. In practice, each of them is shaped by sample preparation, matrix behaviour, screening design, confirmatory evidence, and the quality-control framework used for interpretation.

4.4.2.2 Sensitivity

In EMBRACE, sensitivity is understood as the ability of the analytical workflow to support reliable detection and identification of a toxin at low levels under the applicable qualitative decision rule. This is broader than instrumental sensitivity alone. It includes the contribution of sample preparation, extraction or enrichment efficiency, analyte stability, matrix effects, chromatographic behaviour, and the strength of confirmatory evidence available for the analyte in question.

Because the project is qualitative in purpose, sensitivity is not interpreted primarily as the lowest concentration that can be reported numerically, but as the lowest level at which the workflow can still support a defensible identification decision. For this reason, detection limits, detection capability, or equivalent sensitivity estimates remain necessary even where the final reporting format is not quantitative. In practical terms, the relevant sensitivity may differ between analytical branches: for example, between HILIC-based screening of highly polar low-molecular-weight toxins, RP-LC–MS analysis of less polar analytes, and immunological, functional, or peptide-level confirmation of protein toxins.

4.4.2.3 Specificity and selectivity

Specificity and selectivity refer to the ability of the workflow to distinguish target-related analytical evidence from background signal, structurally related non-targets, and matrix-derived interferences. This is a particularly important requirement in EMBRACE because the intended sample space is broad and includes many matrices capable of generating nonspecific signals or analytical suppression.

In the low-molecular-weight branch, selectivity is achieved through combinations of chromatographic retention, polarity-appropriate separation mode, accurate mass, expected adduct or charge-state behaviour, product-ion evidence, and targeted follow-up where required. In the protein-toxin branch, selectivity may derive from different combinations of evidence, including toxin-specific immunoreactivity, functional activity readouts, selective enrichment, and peptide-level structural confirmation. Accordingly, selectivity is not expressed through one universal analytical criterion across the full workflow, but through branch-appropriate combinations of independent evidence.

Specificity is especially important in a qualitative identification framework because screening alone is not sufficient for high-confidence assignment. The EMBRACE strategy therefore treats specificity not as a property of a single measurement event, but as an evidential outcome supported by orthogonal confirmation where needed.

4.4.2.4 Robustness

Robustness refers to the stability of the qualitative analytical outcome when the workflow is applied under realistic rather than idealised conditions. In EMBRACE, this includes variation in matrix type, sample burden, operator, day of analysis, reagent batch, and instrument conditions. A workflow that performs well only in neat standards or narrowly controlled matrices is not sufficient if the intended use includes unknown environmental, food, biological, or surface-derived samples.

Robustness is therefore one of the most important performance characteristics for assessing analytical readiness in EMBRACE. In the low-molecular-weight branch, robustness is linked especially to the ability of the common intake and routing concept to tolerate diverse matrices without loss of broad screening capability. In the protein-toxin branch, robustness is more strongly affected by enrichment efficiency, reagent behaviour, preservation of toxin activity, and matrix-dependent interference. In both branches, robustness depends not only on analytical chemistry but also on the quality-control framework used to detect invalid or non-interpretable runs.

4.4.2.5 Throughput and deployability as supporting properties

Although not treated as core analytical performance characteristics in the strict sense, throughput and deployability remain important supporting properties of the EMBRACE workflow. The strategy must be capable of handling diverse matrices without excessive fragmentation into separate toxin-specific procedures, and it must remain compatible with realistic laboratory operation. Similarly, deployability is understood as a continuum: rapid screens and field-oriented tools may support prioritisation and early triage, whereas laboratory workflows provide the main confirmatory evidence base. These factors do not replace sensitivity, selectivity, or robustness, but they influence how the workflow can be applied in practice.

4.4.2.6 Combined interpretation

A key point in EMBRACE is that these performance characteristics are not interpreted independently. Sensitivity without selectivity is insufficient for defensible identification, and selectivity without robustness is of limited practical value in real samples. Performance is therefore judged by the combined ability of the workflow to detect toxins at relevant low levels, distinguish them from

interferences, and maintain valid qualitative outcomes across realistic analytical variation. This combined interpretation is what makes the performance concept suitable for a broad, matrix-aware, and qualitative toxin-identification strategy.

4.4.3 Matrix effects, robustness, and sample-related constraints

4.4.3.1 Overview

Matrix effects represent the main practical source of analytical uncertainty across both the low-molecular-weight and protein-toxin branches. This follows directly from the intended sample space, which includes waters, beverages, biofluids, milk, shellfish and food homogenates, soils and sediments, wipe or swab extracts, bloom-associated materials, and other heterogeneous matrices. In such samples, the dominant performance risk is often not lack of intrinsic detector sensitivity, but interference from the sample matrix with extraction, enrichment, chromatographic behaviour, ionisation, immunoreactivity, or functional readout. Matrix behaviour must therefore be regarded as a central design constraint rather than as a secondary validation detail.

4.4.3.2 Matrix effects in the low-molecular-weight branch

In the low-molecular-weight branch, matrix effects arise at several points in the workflow. The first is the common intake and partitioning stage, where analytes are directed into organic and aqueous fractions. Recovery may be influenced by sample composition, incomplete phase separation, adsorption, or co-extraction of interfering material. The second is the chromatographic stage, where highly polar analytes such as paralytic shellfish toxins and less polar analytes such as microcystins, trichothecenes, and alkaloids have markedly different retention requirements and matrix susceptibilities. The third is the ionisation stage, where co-extracted salts, lipids, proteins, pigments, humic components, or other matrix constituents may suppress or enhance electrospray response.

These effects are especially relevant because the low-molecular-weight workflow has been designed for broad class coverage rather than for maximal analyte-specific cleanup. A highly selective preparation may improve performance for one known target while simultaneously reducing the likelihood of detecting related, modified, or unexpected compounds. For this reason, sample preparation has been kept deliberately limited and purposeful. Matrix reduction is introduced where necessary, but not to the extent that the breadth of screening is unnecessarily compromised. Robustness in this branch therefore depends on achieving a workable balance between matrix control and analyte coverage, not on complete removal of all interfering material.

4.4.3.3 Matrix effects in the protein-toxin branch

In the protein-toxin branch, matrix effects are expressed through somewhat different mechanisms. Complex matrices may impair antibody binding in immunoassays, reduce affinity-capture efficiency, increase nonspecific background, inhibit enzymatic or functional readout, or complicate peptide-level confirmation after digestion. In functional assays, the matrix may directly influence the activity being measured. In peptide-level methods, rich protein backgrounds may reduce sensitivity unless selective enrichment is applied before digestion and LC–MS/MS analysis.

These effects are not merely theoretical. In the current protein-toxin workflows, front-end handling has already been recognised as critical in matrices such as serum, milk, soil/sand, and fecal material for ricin analysis, and similarly in serum and other complex materials for botulinum neurotoxin assays. Matrix burden therefore affects not only analytical signal, but also which evidential layer can be applied most effectively. Stronger cleanup is not automatically the solution, since excessive processing may reduce recovery, alter functional integrity, or remove material needed for confirmation. The central requirement is therefore to reduce matrix burden while preserving the evidential value of the downstream method.

4.4.3.4 Sample-related constraints beyond matrix effects

Analytical performance is also constrained by sample-related factors beyond matrix composition alone. One of these is limited sample amount. A broad screening workflow ideally allows progression into both the low-molecular-weight and protein-toxin branches, with reserve material available for orthogonal confirmation. In practice, restricted sample volume or mass may limit the number of branches or follow-up steps that can be applied. Under such conditions, the routing logic must preserve the highest possible evidential value without assuming that all analytical options can be applied simultaneously.

A second constraint is sample heterogeneity. Powders, soils, sediments, shellfish homogenates, bloom materials, and other particulate-rich samples may be compositionally non-uniform, so that subsampling and homogenisation become important determinants of apparent method performance. In such cases, variability may arise before instrumental analysis begins. Robustness must therefore be considered at the level of the full workflow, including pre-analytical handling, rather than only at the level of the analytical instrument.

A third constraint is the timing of sample collection relative to toxin exposure or toxin release. In clinical or post-exposure settings, direct toxin detection may be limited by degradation, metabolism, redistribution, or clearance. The practical detection window may therefore be narrower than the intrinsic instrumental capability would suggest. This is particularly important when interpreting negative findings from biological matrices.

4.4.3.5 Implications for robustness

Under these conditions, robustness cannot be defined as identical analytical behaviour across all matrices and toxin classes. Rather, robustness must be understood as the ability of the workflow to maintain valid and interpretable qualitative outcomes despite realistic variation in sample type, matrix burden, handling conditions, and analytical context. This is a more demanding requirement than performance in neat standards or simplified laboratory matrices, but it is the appropriate standard for a workflow intended for unknown samples.

For this reason, robustness depends not only on instrument performance, but also on the combined effect of broad matrix grouping, branch-specific routing, purposeful sample preparation, and

orthogonal confirmation. These are all parts of the analytical response to the risks introduced by matrix effects and variable sample quality.

4.4.3.6 Management of matrix-related risk

Matrix-related uncertainty is managed through several linked measures. Samples are first assigned to broad operational matrix classes rather than narrowly defined categories. Sample preparation is then kept deliberate rather than excessive, so that matrix burden is reduced without unnecessarily sacrificing analyte breadth or evidential integrity. Separate hydrophilic and reversed-phase LC–MS channels are used where chromatographic behaviour requires it. In the protein-toxin branch, selective enrichment and functional or peptide-level follow-up are used where screening alone is insufficient. Finally, blanks, matrix controls, spiked controls, surrogate or internal controls where appropriate, and run-acceptance criteria are used to identify invalid or non-interpretable analytical outcomes.

Overall, matrix effects and sample-related constraints are best regarded as expected properties of the intended sample space rather than as exceptional complications. The workflow has therefore been designed on the assumption that qualitative identification must remain defensible under imperfect and variable sample conditions, and that reliable interpretation depends as much on controlled handling and evidential structure as on instrumental capability alone.

4.4.4 QA/QC, acceptance criteria, and qualitative decision rules

4.4.4.1 Purpose of the QA/QC framework

Quality assurance and quality control provide the basis for defensible qualitative identification. Because the workflow is intended for broad toxin screening in unknown samples, QA/QC must support not only instrument performance but also sample preparation, matrix handling, enrichment behaviour, functional response, and interpretation of analytical evidence. The purpose of the QA/QC framework is therefore to demonstrate that a given analytical result was produced under conditions that were fit for purpose and that the resulting identification decision is supported by valid analytical evidence (Ellison & Bettencourt da Silva, 2021; Magnusson & Örnemark, 2014).

In this context, QA/QC is not limited to detecting instrument malfunction. It must also reveal contamination, carry-over, matrix suppression or enhancement, poor enrichment efficiency, nonspecific signal, functional inhibition, and other conditions that may compromise interpretation. A result is therefore considered valid only if the relevant blanks, controls, and module-specific acceptance criteria indicate that the analytical run can support a qualitative decision (Ellison & Bettencourt da Silva, 2021).

4.4.4.2 Qualitative rather than quantitative QA/QC objective

The workflow is intended as a general cross-class screening procedure. It follows necessarily that full quantitative performance, including uniform analyte recovery across all analytes and matrices, is neither expected nor required as a routine objective. The purpose of QA/QC is not to demonstrate

that every analyte is recovered quantitatively from every sample type, but to show that the workflow remains capable of supporting reliable qualitative identification. This is consistent with fitness-for-purpose principles for non-quantitative analytical use (Ellison & Bettencourt da Silva, 2021; Magnusson & Örnemark, 2014).

For this reason, QA/QC is focused primarily on whether the analytical evidence remains interpretable and whether the lower limit of qualitative detection is known, at least approximately, for the analytes and modules concerned. Approximate understanding of analyte loss, matrix-related suppression, or incomplete recovery remains useful during development, but these are interpreted mainly in relation to detectability and identification capability rather than concentration reporting (Ellison & Bettencourt da Silva, 2021).

4.4.4.3 Core QA/QC elements

Across the workflow, several QA/QC elements recur irrespective of toxin class. Procedural and reagent blanks are used to monitor contamination, carry-over, and laboratory background. Matrix blanks or negative matrix controls are used to determine whether the matrix itself generates analytical response that may interfere with interpretation. Fortified controls or spiked matrix samples are used to assess whether analytical signal remains detectable in the presence of the matrix and to reveal marked suppression, masking, or inhibition. Internal standards, surrogate markers, or equivalent system-suitability controls are used where applicable to monitor extraction, chromatography, or signal generation (Ellison & Bettencourt da Silva, 2021; Magnusson & Örnemark, 2014).

In the low-molecular-weight branch, these QA/QC elements must demonstrate that the selected preparation route, chromatographic channel, and mass-spectrometric evidence are suitable for the sample type examined. In the protein-toxin branch, they must additionally support interpretation of immunological specificity, enrichment performance, functional response, and peptide-level confirmation. The exact control design differs between workflow elements, but the principle remains the same throughout: no positive, negative, or inconclusive decision should be reported unless the analytical conditions that produced it can be shown to have been acceptable (Ellison & Bettencourt da Silva, 2021).

4.4.4.4 Acceptance criteria at workflow level

Acceptance criteria determine whether analytical data are valid for interpretation. In a qualitative workflow, they should not be reduced to a single numeric threshold. Rather, they define whether the evidential quality of the run is sufficient to support classification of a result as identified, not identified, or analytically inconclusive. This is consistent with the statistical framework of decision threshold and detection capability and with later methodological discussion on combining qualitative identification criteria with CCα/CCβ concepts (European Commission, 2002; Van Loco et al., 2007).

At minimum, acceptance criteria should address blank behaviour, control performance, system suitability, and branch-specific evidence quality. In LC–MS-based workflows, this includes acceptable blank status, expected retention behaviour, adequate signal quality, and appropriate precursor/product-ion or transition evidence. In immunological assays, it includes acceptable

behaviour of negative controls, positive controls, spike controls, and, where applicable, irrelevant-capture controls used to identify nonspecific matrix reactivity. In functional assays, acceptance criteria must also address whether the observed signal is meaningfully above background and whether the assay context supports attribution of that signal to the intended toxin mechanism (Ellison & Bettencourt da Silva, 2021).

4.4.4.5 Qualitative decision rules

Whereas acceptance criteria determine whether data are valid for interpretation, decision rules define how valid data are classified. The analytical framework is based on predefined qualitative decision rules for distinguishing between identified, not identified, and analytically inconclusive outcomes. Because the workflow does not aim at concentration reporting, identification decisions must be based on the weight and coherence of the available analytical evidence rather than on one universal numeric cutoff across all analytes, matrices, and evidential forms (Ellison & Bettencourt da Silva, 2021).

In the low-molecular-weight branch, a presumptive identification is supported by agreement between expected chromatographic behaviour, precursor mass or transition, product-ion evidence, adduct or charge-state plausibility, and acceptable control performance. Higher-confidence identification requires confirmation through a second analytical dimension or more selective follow-up, for example targeted QqQ analysis after HRMS screening or concordant evidence from multiple mass-spectrometric parameters. In the protein-toxin branch, the same logic applies in broader form, with immunological recognition, functional activity, selective enrichment, and peptide-level structural confirmation contributing complementary evidence. Screening signal alone is generally insufficient for definitive identification where evidential requirements are higher.

Thus, the decision rule is not simply whether signal is present, but whether the available evidence satisfies the predefined criteria for a supported identification. Where evidence is incomplete, contradictory, or compromised by control failure, the conclusion should remain limited rather than overstated.

4.4.4.6 Decision threshold (CCα) and detection capability (CCβ)

For qualitative identification, the established framework based on decision threshold and detection capability is adopted. The decision threshold, also referred to as the critical level, is the response or evidence boundary above which a result is classified as positive at a predefined false-positive probability α. In IUPAC/ISO terminology, this corresponds to LCL/CLC or CCα. The detection capability is the lowest analyte level at which the method classifies the sample as positive with probability (1 – β), given the predefined identification criteria. In IUPAC/ISO terminology, this corresponds to LDL/DLD or CCβ (European Commission, 2002; ISO, 1997, 2019).

In the present workflow, these parameters are linked to the full qualitative decision rule rather than to a purely instrumental signal cutoff. **CCα** defines the minimum evidential boundary required for a positive identification decision, whereas **CCβ** defines the lowest analyte level at which that decision can be achieved with the required confidence. This framework is appropriate because it allows qualitative presence/absence decisions to be expressed statistically without converting the workflow into a quantitative reporting method (European Commission, 2002; Van Loco et al., 2007).

Where full CCα/CCβ determination is not yet feasible for a given analyte or module, a practical lower limit of qualitative detection should at minimum be estimated, since this determines whether the analyte remains identifiable after passage through the general screening workflow (Ellison & Bettencourt da Silva, 2021).

4.4.4.7 Inconclusive and non-interpretable outcomes

A robust qualitative reporting framework must also accommodate outcomes that do not justify a binary positive-or-negative conclusion. In practice, some results will be analytically inconclusive because the observed signal is suggestive but not sufficiently supported, whereas others will be non-interpretable because matrix behaviour, control failure, insufficient sample amount, or other analytical limitations prevent reliable evaluation. Qualitative-analysis guidance explicitly supports such graded interpretation because it prevents overstatement of evidence and makes analytical limitations visible (Ellison & Bettencourt da Silva, 2021).

These categories are essential in a broad toxin-screening workflow. Accordingly, the reporting logic should distinguish, where appropriate, between no toxin identified, toxin-related signal detected but not conclusively identified, toxin identified under the predefined decision criteria, and result not interpretable because of analytical limitation.

4.4.4.8 Relationship to OPCW-oriented reporting criteria

The qualitative decision framework should also be understood in relation to emerging OPCW-oriented reporting expectations. Trial proficiency-test reporting instructions have already defined toxin-specific evidential criteria for at least a low-molecular-weight saxitoxin/PST-related scenario and a high-molecular-weight ricin scenario. These include explicit expectations regarding use of reference materials or reference data, same-run blanks and controls, signal-to-noise or equivalent evidential thresholds, and, in the ricin case, peptide-level confirmation requirements extending across both A and B chains (OPCW Technical Secretariat, 2024, 2025).

These criteria provide an important reference point for verification-oriented reporting and show that identification expectations may differ by toxin class and analytical module. However, they remain scenario-specific and should not yet be interpreted as a final universal reporting framework for the full toxin panel. For this reason, a general qualitative QA/QC and decision-rule structure is adopted here, while allowing toxin-class-specific evidential criteria to be defined where required.

4.4.4.9 Interpretation of negative findings

Because the workflow is qualitative, a negative result must also be interpreted within the QA/QC framework. Non-detection does not imply absolute absence of toxin; it means that no toxin was identified under the analytical conditions, sensitivity, and decision criteria applied. This is especially important in complex matrices, limited samples, or late post-exposure materials, where the practical detection window may be constrained. Qualitative-analysis guidance and method-fitness guidance both support this more limited interpretation of non-detection (Ellison & Bettencourt da Silva, 2021; Magnusson & Örnemark, 2014).

4.4.4.10 Operational role in the analytical pathway

At workflow level, QA/QC, acceptance criteria, and qualitative decision rules connect analytical performance to reporting. They ensure that broad screening remains defensible even when samples are heterogeneous, matrices are difficult, and different analytical branches generate different forms of evidence. They also allow one integrated analytical strategy to be maintained without requiring identical criteria for every toxin class. Instead, branch-specific and toxin-specific criteria can be applied within a common quality framework.

Overall, the purpose of this framework is to ensure that identification claims are explicit, reviewable, and supported by controlled analytical evidence, while making analytical limitation visible where full identification is not justified.

4.4.5 Key qualitative performance terms and statistical concepts

4.4.5.1 Purpose of the definitions

The terms below are included to support consistent discussion of method readiness, quality-control interpretation, and evidential strength across the analytical workflow. Some of these terms originate from broader analytical validation practice, including quantitative method validation, but they remain useful here insofar as they describe variability, matrix influence, recovery, sensitivity, and reliability of the analytical evidence. In the present context, they are used to support qualitative toxin identification and readiness assessment rather than routine concentration reporting (Ellison & Bettencourt da Silva, 2021; Magnusson & Örnemark, 2014).

Because the overall workflow is designed as a general cross-class screening procedure, these definitions should not be interpreted as implying that every analyte will be handled through a fully quantitative method with optimised recovery. Instead, the key requirements are that approximate analyte behaviour is understood and that the lower limit of qualitative detection is established, at least at an estimated level, for the analytes and modules covered (Ellison & Bettencourt da Silva, 2021).

4.4.5.2 Decision threshold (CCα)

The concepts of decision threshold (CCα) and detection capability (CCβ) are introduced in Section 4.4.4.6 in the context of qualitative decision rules. The definitions provided here formalise the terminology for consistent use in reporting, QA/QC interpretation, and method-characterisation discussions.

The decision threshold (CCα) is the response or evidence boundary above which a result is judged positive at a predefined false-positive probability α. In IUPAC/ISO terminology, this corresponds to the critical level (LCL/CLC) (European Commission, 2002; ISO, 1997).

Within the present workflow, CCα represents the minimum evidential boundary required for a sample to be classified as identified or present under the adopted qualitative decision rule.

4.4.5.3 Detection capability (CCβ)

The detection capability (CCβ) is the lowest analyte level at which the method identifies the analyte with probability (1 − β), given the predefined identification criteria. In IUPAC/ISO terminology, this corresponds to the minimum detectable value (LDL/DLD) (European Commission, 2002; ISO, 2019).

Within the present workflow, CCβ represents the lowest analyte level at which the full qualitative identification decision can still be made with the required confidence.

4.4.5.4 Practical role of CCα and CCβ in the workflow

Taken together, CCα and CCβ provide the principal statistical framework for qualitative analytical sensitivity. CCα defines when the available analytical evidence becomes sufficient to support a positive decision, whereas CCβ defines the analyte level at which that decision can be made reliably. For the present workflow, their practical importance lies in defining the lower boundary at which qualitative identification remains defensible (Ellison & Bettencourt da Silva, 2021).

4.4.5.5 Limit of detection and sensitivity estimates

Although the intended analytical use is qualitative, a defined understanding of analytical sensitivity is still required. Where authentic reference standards are available, analyte-specific estimates of detection limit or detection capability can be established directly for the relevant matrix and analytical module. Where standards are unavailable, practical sensitivity may need to be approximated using structurally related reference compounds, representative congeners, surrogate materials, or evidence-based identification thresholds derived from the screening workflow. Qualitative-analysis guidance recognises that such estimated thresholds may still be useful and necessary for classification-based methods (Ellison & Bettencourt da Silva, 2021). A broader conceptual basis for detection-limit language is also provided by Currie (1999).

Accordingly, the absence of a quantitative reporting objective does not remove the need for sensitivity assessment. It changes its purpose from concentration assignment to defensible qualitative detection and identification.

4.4.5.6 Precision in a qualitative method

In a qualitative workflow, precision is assessed primarily through the repeatability of the analytical evidence and the resulting identification outcome rather than through repeatability of a reported concentration value. This may include consistency of retention behaviour, mass accuracy, product-ion or transition evidence, immunological response, functional signal, peptide detection, or other branch-specific evidence, as well as the consistency with which replicate samples are classified under the predefined decision rule.

Precision is therefore the closeness of agreement between independent test results obtained under specified conditions, interpreted here mainly as the consistency of the analytical evidence and of the resulting qualitative classification (Ellison & Bettencourt da Silva, 2021).

4.4.5.7 Repeatability

Repeatability is precision under repeatability conditions, where independent test results are obtained using the same method on identical test items in the same laboratory, by the same operator, using the same equipment, within a short period of time (Ellison & Bettencourt da Silva, 2021).

4.4.5.8 Intra-assay repeatability

Intra-assay repeatability is precision within a single analytical run under otherwise constant conditions.

4.4.5.9 Inter-assay repeatability

Inter-assay repeatability is precision between analytical runs performed under similar conditions, typically on different days, with only limited variation in factors such as reagent batch or operator.

4.4.5.10 In-house reproducibility

In-house reproducibility is precision under conditions in which the method is performed repeatedly within the same laboratory, but with variation in one or more factors such as operator, day of analysis, reagent batch, or instrument.

4.4.5.11 Reproducibility

Reproducibility is precision under reproducibility conditions, where test results are obtained with the same method on identical test items in different laboratories, with different operators and different equipment.

4.4.5.12 Replicates

Replicates may be divided into **independent sample replicates**, **preparation replicates**, and **measurement replicates**. Independent sample replicates are separate sample units or separately fortified matrix aliquots representing the same condition. Preparation replicates are parallel subsamples processed independently through the analytical workflow. Measurement replicates are repeated measurements made from the same prepared extract or reaction mixture, for example replicate LC–MS injections or duplicate immunoassay wells. (Ellison & Bettencourt da Silva, 2021).

4.4.5.13 Matrix effect

A matrix effect is the alteration of analytical response caused by co-occurring sample components rather than by the analyte itself. In LC–MS workflows this often appears as ion suppression or ion enhancement, but in broader analytical terms it may also include impaired antibody binding, reduced enrichment efficiency, elevated nonspecific background, or inhibition of functional readout. Qualitative-analysis guidance explicitly treats matrix influence as part of method performance and uncertainty (Ellison & Bettencourt da Silva, 2021).

4.4.5.14 Recovery

Recovery describes the fraction of analyte retained through the sample-preparation process relative to an appropriate reference condition. In the present context, recovery is used mainly as a development and readiness parameter rather than as a reporting parameter. Its main purpose is to indicate whether an analyte is likely to remain detectable after the general screening workflow has been applied. Depending on the workflow, recovery may be evaluated through pre- and post-

extraction fortification, pre- and post-enrichment comparison, or another experimentally justified design (Magnusson & Örnemark, 2014).

4.4.5.15 Process efficiency

Process efficiency is a measure that includes both sample-preparation recovery and matrix effects during measurement. To determine process efficiency for a toxin in a given matrix, the analytical response or measured concentration obtained from an equivalent fortification in buffer may be taken as 100%, and the measured result obtained in the matrix is expressed relative to that value as a percentage. In the present workflow, process efficiency is used mainly to judge whether matrix-related loss is compatible with continued qualitative detection (Magnusson & Örnemark, 2014).

4.4.5.16 Robustness

Robustness is the ability of the workflow to maintain valid and interpretable qualitative outcomes despite realistic variation in matrix type, sample burden, operator, day of analysis, reagent batch, or instrument conditions. In the present context, robustness is judged at the level of the overall analytical pathway rather than solely at the level of one detector response (Ellison & Bettencourt da Silva, 2021; Magnusson & Örnemark, 2014).

4.4.5.17 Accuracy and trueness

Accuracy is the closeness of agreement between a test result and an accepted reference value. Trueness is the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. Within the present qualitative workflow, these terms are of secondary importance relative to CCα, CCβ, detection capability, selectivity, and robustness. They remain relevant mainly during development-stage evaluation using fortified samples, reference materials, or method-comparison exercises, where they help describe analytical readiness even though the final routine reporting format is not quantitative (Magnusson & Örnemark, 2014).

4.4.5.18 Interpretive role in the workflow

Taken together, these concepts provide the terminology needed to assess whether a workflow element is fit for qualitative toxin identification. In the present context, they are not intended to impose a fully quantitative validation model on every analytical module. Rather, they support a structured and statistically grounded approach to readiness assessment, quality control, and evidential interpretation within a broad, matrix-aware, and qualitative toxin-identification strategy (Ellison & Bettencourt da Silva, 2021).

4.5 Reporting principles and evidential standards

4.5.1 Purpose of reporting

4.5.1.1 Reporting objective

The purpose of reporting is to communicate analytical findings in a form that is scientifically defensible, operationally useful, and compatible with investigation-oriented requirements. Because

the analytical strategy is qualitative in purpose, reporting is centred on **identification**, **non-identification**, or **analytical limitation**, rather than on routine concentration reporting. This is consistent with the broader qualitative-analysis framework discussed above and with the emphasis placed by both OPCW and UNSGM-related materials on controlled, reviewable analytical conclusions rather than unrestricted scientific interpretation (Ellison & Bettencourt da Silva, 2021; OPCW Scientific Advisory Board [SAB], 2023; United Nations Office for Disarmament Affairs [UNODA], 2023).

4.5.1.2 Reporting as part of the evidential chain

Reporting is treated as part of the overall evidential chain rather than as a final administrative step. For that reason, a reported conclusion should reflect not only the observed analytical result but also the analytical route taken, the quality controls applied, the evidential basis for identification, and the principal limitations affecting interpretation. This is particularly important in an investigation context, where laboratory reports must differ from open-ended scientific discussion and should instead support traceable and reviewable decision-making (UNODA, 2023).

4.5.2 General reporting philosophy

4.5.2.1 Qualitative and evidence-based reporting

Results should be reported within a qualitative, evidence-based framework. The principal reporting question is whether the available analytical evidence is sufficient to support classification of a toxin as identified, not identified, or not interpretable under the predefined decision rule. This follows the same logic as the CCα/CCβ framework discussed above and is consistent with the Eurachem/CITAC approach to qualitative analysis (Ellison & Bettencourt da Silva, 2021; European Commission, 2002; ISO, 1997, 2019).

4.5.2.2 No routine concentration reporting

Routine reporting of concentrations is not required for the intended use of the workflow. This follows directly from the fact that the overall screening procedure is broad, cross-class, and not optimised for uniform quantitative recovery of every analyte in every matrix. Nevertheless, reporting should still reflect the known or estimated analytical sensitivity of the method, especially the lower limit of qualitative detection or detection capability where available. Negative findings should therefore be reported in a qualified manner, that is, as non-identification under the applied method conditions and decision criteria, rather than as proof of absolute absence (Ellison & Bettencourt da Silva, 2021; Magnusson & Örnemark, 2014).

4.5.2.3 Conservative reporting logic

Where evidence is incomplete, contradictory, or affected by control failure or matrix interference, conclusions should remain appropriately limited. In such cases, the correct reporting outcome is not forced attribution but a statement of inconclusive evidence or analytical limitation. This approach is consistent with qualitative-analysis guidance and with verification-oriented expectations that emphasize reliability, transparency, and defensible interpretation over overstatement (Ellison & Bettencourt da Silva, 2021; OPCW SAB, 2023).

4.5.3 Minimum reporting content

At minimum, the report for a given sample or sample set should include the following elements:

4.5.3.1 Sample and matrix description

The report should state the sample identifier, relevant chain-of-custody or receipt identifier where applicable, and the matrix description used for analytical routing. This is consistent with UNSGM-oriented emphasis on traceability, chain of custody, and structured sample handling and analysis (UNODA, 2023).

4.5.3.2 Analytical modules applied

The report should specify which analytical branch or branches were applied, for example low-molecular-weight LC–MS screening, immunological screening, functional assay, peptide-level confirmation, or targeted follow-up. This is important because the evidential value of a result depends in part on the analytical module used and on whether orthogonal confirmation was achieved (OPCW SAB, 2023; OPCW SAB, 2021).

4.5.3.3 Result category

The final result should be expressed in a controlled reporting category, such as:

- toxin identified,
- toxin not identified,
- toxin-related signal detected but not conclusively identified,
- result not interpretable because of analytical limitation.

This kind of graded reporting is consistent with qualitative analytical guidance and is preferable to a forced binary statement where evidential support is incomplete (Ellison & Bettencourt da Silva, 2021).

4.5.3.4 Basis of identification

For any identified toxin, the report should state the analytical basis for the identification. Depending on the workflow, this may include chromatographic behaviour, precursor/product-ion evidence, targeted transitions, immunological recognition, functional response, peptide-level confirmation, or other orthogonal evidence. The report should make clear whether the conclusion is based on one evidential layer or on concordant evidence from more than one analytical dimension (OPCW SAB, 2023).

Quality-control basis

The report should state, either directly or through reference to controlled analytical records, that the applicable blanks, controls, and acceptance criteria were met. Where a result is limited by control failure or matrix behaviour, this should be stated explicitly (Ellison & Bettencourt da Silva, 2021).

Limitations and uncertainty

Any major limitations affecting interpretation should be declared. These may include limited sample amount, matrix interference, estimated rather than standard-backed detection capability, lack of an authentic reference standard, or incomplete orthogonal confirmation. This is important because qualitative identification should be accompanied by explicit recognition of evidential strength and limitation rather than by an implied claim of unrestricted certainty (Ellison & Bettencourt da Silva, 2021; Pereira, 2022).

4.5.4 Reporting categories and interpretive wording

Identified

A toxin should be reported as **identified** only when the predefined evidential criteria for that toxin class and analytical module have been satisfied. In practice, this means that the analytical evidence must cross the applicable decision threshold and satisfy the relevant identification criteria, including acceptable control performance. Where the workflow requires orthogonal confirmation for decision-grade identification, the report should indicate that such confirmation was achieved (European Commission, 2002; Ellison & Bettencourt da Silva, 2021).

Not identified

A toxin should be reported as **not identified** when the analytical evidence does not support identification under the applied workflow and decision rule. This wording is preferable to "absent," because it correctly reflects the fact that non-detection is conditional on the method used, the matrix, the sample condition, and the practical detection capability of the workflow (Ellison & Bettencourt da Silva, 2021).

Detected but not conclusively identified

This category is appropriate when toxin-related analytical evidence is present, but the result does not satisfy the full criteria required for identification. Examples may include partial mass-spectrometric evidence, immunological signal without orthogonal support, or suggestive peptide evidence below the reporting threshold for confirmation. Such wording preserves potentially important intelligence or investigational value without overstating evidential certainty (Ellison & Bettencourt da Silva, 2021; OPCW SAB, 2023).

Not interpretable

This category is appropriate where control failure, matrix effects, insufficient sample amount, analyte instability, or other analytical limitations prevent reliable interpretation. This category is especially important in broad-scope toxin analysis, because it makes the boundary between a true negative and an analytically compromised result explicit (Ellison & Bettencourt da Silva, 2021).

4.5.5 Orthogonal evidence and toxin-class-specific evidential standards

General requirement for orthogonality

Orthogonal evidence should be reflected explicitly in the reporting standard. The need for this is strongly supported by OPCW biotoxin work, which has emphasised the use of complementary analytical dimensions rather than single-method claims, particularly for high-molecular-weight biotoxins, where immunological, functional, and mass-spectrometric evidence may all contribute to defensible identification (OPCW SAB, 2023; OPCW SAB, 2021).

Toxin-class-specific criteria

At the same time, reporting criteria should not be assumed to be fully identical across all toxin classes. OPCW work on biotoxins has explicitly recognised the need for common guidelines and best practices while also acknowledging that requirements for acceptable data and reporting criteria may need to be harmonised further between the OPCW and the UN in relation to biotoxins (OPCW SAB, 2023; OPCW SAB, 2025). Current OPCW trial proficiency-test work has also proceeded through toxin-specific scenarios rather than through one universal biotoxin rule set (OPCW SAB, 2025).

Accordingly, the reporting standard should combine:

1. a **common general reporting framework** for all toxins, and
2. **toxin-class-specific evidential criteria** where required.

This allows one consistent reporting philosophy to be maintained while recognising that, for example, the evidential structure for paralytic shellfish toxins differs from that for ricin or botulinum neurotoxins.

4.5.6 Relationship to UNSGM- and OPCW-oriented expectations

UNSGM-oriented considerations

UNSGM-related materials emphasise that analytical laboratories *supporting investigations must operate within a broader framework that includes chain of custody, controlled sample handling, quality assurance, method validation, and reporting requirements*. They also explicitly distinguish investigation reporting from ordinary scientific reporting and treat report writing, confidentiality, and structured analytical capability as part of operational readiness (UNODA, 2023; Grunow, 2018). This supports the inclusion of a dedicated reporting framework in the present deliverable rather than leaving reporting as an implicit by-product of analytical method development.

OPCW-oriented considerations

OPCW Scientific Advisory Board work on biotoxins has similarly highlighted the need for common guidelines and best practices for biotoxin analysis, coordination of quality-assurance requirements, and harmonisation of analytical techniques and reporting criteria requirements between the OPCW and the UN in relation to biotoxins (OPCW SAB, 2023; OPCW SAB, 2025). The OPCW biotoxin programme has also placed particular emphasis on confidence-building exercises using ricin and

saxitoxin-related scenarios as a basis for building common analytical and reporting practice (OPCW SAB, 2023; OPCW SAB, 2025).

Implication for the present deliverable

The reporting standard proposed here should therefore be understood as compatible with these emerging investigation-oriented expectations, while not claiming that a final universal international reporting standard for all biotoxins already exists. Instead, the current deliverable can reasonably define:

- the **general reporting philosophy**,
- the **minimum reporting content**,
- the **controlled reporting categories**, and
- the **principle of toxin-class-specific evidential criteria**.

That is an appropriate level of definition for the present stage of method development.

4.5.7 Overall reporting principle

Overall, reporting should be **qualitative, evidence-based, conservative, and explicit about limitation**. A report should state what was done, what was found, what level of identification was achieved, what controls support that conclusion, and what limitations remain. In this way, reporting becomes the final controlled expression of the analytical strategy rather than a loosely worded summary of instrument outputs. This is consistent with both qualitative analytical best practice and with the broader expectations emerging from UNSGM- and OPCW-related work on investigation-ready analytical capability (Ellison & Bettencourt da Silva, 2021; OPCW SAB, 2023; UNODA, 2023).

5 GAPS, CONSTRAINTS AND READINESS ASSESSMENT

The first-iteration analytical scope is intentionally limited to the toxin targets listed in the Description of Action. In the present deliverable, these targets are treated as **canonical exemplars of broader toxin classes**, rather than as the full long-term analytical scope. The purpose of the current iteration is therefore not to establish complete coverage of all possible biotoxins, but to define and develop defensible analytical workflows for representative targets that anchor later expansion. This is consistent with the current project framing, which already treats the selected targets as the basis for broader method families and later ROP development rather than as a closed final panel.

At the same time, analytical readiness must be considered separately for two related but distinct objectives. The first is **qualitative detection and identification readiness**, that is, whether a target can be screened and, where applicable, confirmed within a defined analytical scope. The second is **attribution-oriented analytical readiness**, meaning the extent to which the workflow already supports later provenance-related interpretation through features such as impurity patterns, purity-related observations, or variant discrimination. In the present iteration, the first objective is substantially more advanced than the second. Current work has already identified attribution-relevant analytical dimensions, including impurity profiling approaches to support provenance analysis, but no target currently has an attribution-ready workflow within the EMBRACE framework.

5.1 Purpose and approach

This section summarises the principal gaps, constraints, and current analytical readiness for the targets addressed in Task 3.2. The purpose is to indicate, in a transparent and decision-useful way, what level of analytical output is currently achievable within the present framework, what remains under development, and which dependencies are most likely to limit further progress.

Readiness is assessed against the analytical philosophy set out in this deliverable, particularly:

- the distinction between **screening/triage** and **confirmatory identification**;
- the requirement for **orthogonal evidence** in decision-grade identification;
- the need for **matrix-aware scope statements**;
- the requirement for **documented QA/QC, decision rules, and reporting logic** suitable for defensible qualitative reporting; and
- the recognition that **attribution-oriented analysis** is a separate and less mature development track.

Readiness in this context does not mean full validation for all matrices and all possible sample histories. Rather, it refers to the degree to which a target-specific workflow has progressed from conceptual selection to practical implementation within a defined analytical scope.

5.2 Readiness scale used in this deliverable

The analytical readiness framework applied in this deliverable is formalised in Annex C. Annex C defines the meaning of R0–R3, the evidential basis for readiness assignment, and the relationship of readiness to scope, matrix limitations, QA/QC, orthogonality, and reporting.

The following readiness categories are used:

- **R0 — Not initiated**
  - No practical workflow has yet been established in the current iteration.
- **R1 — Screening-ready**
  - A screening or triage capability exists, but the evidential chain is not yet sufficient for confirmatory reporting.
- **R2 — Confirmation under development**
  - A confirmatory workflow has been defined in principle and at least partly implemented, but remains incomplete with respect to matrix scope, evidential chain, QA/QC structure, or reporting criteria.
- **R3 — Confirmation-ready (defined scope)**

Qualitative identification is achievable within a defined scope and for defined matrices, with documented orthogonality, QA/QC logic, and reporting basis suitable for defensible interpretation.

In this deliverable, these readiness categories apply primarily to **detection and identification readiness**. Attribution-oriented readiness is assessed more cautiously and, at the present stage, should generally be understood as **preparatory only**. The analytical features that may later support attribution have begun to be identified, but the comparative materials, representative datasets, and interpretive frameworks needed for robust provenance-oriented analysis are not yet in place.

5.3 Cross-cutting constraints affecting readiness

Across the target panel, readiness is shaped primarily by several common constraints.

Matrix diversity and unknown sample history

The intended sample space includes environmental materials, wipe and swab extracts, powders, foods, biological fluids, and mixed unknowns. In many cases, matrix composition and sample history are uncertain. This affects recovery, selectivity, robustness, and the practical value of a given analytical workflow. Readiness must therefore be interpreted together with clearly stated matrix scope and limitations.

Requirement for orthogonal confirmation

Forensic or decision-grade readiness is not achieved simply because a screening signal can be generated. Readiness depends on whether a sufficient evidential chain exists, including independent confirmatory dimensions where required. This requirement is particularly important for protein toxins and for any analytical context where identification claims must be defensible beyond screening alone.

Availability of reference materials, standards, and reagents

Certified materials, authentic standards, antibodies, and other critical reagents are unevenly available across the panel. This constrains method optimisation, sensitivity estimation, and, in some areas, the pace at which confirmatory workflows can be consolidated.

Single-laboratory implementation in the present iteration

For this stage of the project, analytical development has taken place primarily within one laboratory. Current readiness therefore reflects in-house development and internal verification rather than interlaboratory validation or demonstrated transferability across laboratories.

Qualitative rather than quantitative workflow design

The present workflow is designed for broad qualitative identification rather than analyte-specific quantitative recovery. This is appropriate to the project objective, but it means that readiness depends mainly on detection capability, evidential sufficiency, and reporting logic rather than on fully quantitative validation metrics.

Attribution-oriented constraints

Attribution-oriented work is expected to be substantially more constrained than detection and identification work. Progress in this area depends not only on analytical method development, but also on **access to suitable comparative materials, representative preparations, and sufficiently broad reference datasets** against which analytical features can be interpreted. For several targets, assembling such material is likely to be difficult. Accordingly, the current iteration is best understood as having defined the main **analytical directions for later attribution work**, rather than having established operational attribution capability.

5.4 Target-specific gaps and current readiness

In this first iteration, the readiness assignments below are matrix-sensitive and scope-bound, but they are not presented as an exhaustive matrix-by-matrix scoring exercise. They should be interpreted together with the representative matrices examined, the broad matrix classes used in the workflow, and the stated limitations for each target.

5.4.1 Botulinum neurotoxins

Identification readiness: R1

Confirmatory capability is under development. Important analytical modules have already been defined, including immunological screening, Endopep-type functional detection, and peptide-level confirmation logic. The principal remaining gaps relate to matrix-specific implementation, consolidation of the full evidential chain, and clearer definition of scope and reporting criteria for confirmation-ready use.

Attribution-oriented status: preparatory only.

No provenance-oriented workflow has yet been established.

5.4.2 Ricin

Identification readiness: R3 (defined scope)

Ricin is the most advanced protein-toxin target in the current iteration. Screening, functional, and peptide-level confirmation elements are already available in draft operational form. The main remaining gaps relate to extension of robustness across additional matrices, clearer delimitation of the matrices and conditions within which R3 is claimed, and continued refinement of reporting criteria. The current workflow already reflects the orthogonal confirmation model expected for high-molecular-weight toxins.

Attribution-oriented status: preparatory only.

Potentially relevant analytical features have been recognised, but no attribution-ready workflow is in place.

5.4.3 Paralytic shellfish toxins / saxitoxin-group toxins

Identification readiness: R3 (defined scope)

Confirmation-ready within defined analytical scope, particularly where native-congener HILIC-LC–MS/MS workflows are applicable. The main remaining gaps relate to broader extension into non-standard forensic matrices and continued alignment of reporting criteria with verification-oriented expectations. The OPCW reporting criteria for the low-molecular-weight toxin trial provide a useful reference point for evidential expectations, but they do not by themselves resolve the wider matrix-scope question.

Attribution-oriented status: not yet established.

5.4.4 Aconitum alkaloids

Identification readiness: R2

Confirmatory capability is under development. The principal analytical route is clear and well supported by LC–MS/MS methodology, but remaining gaps include broader panel completion, matrix robustness across intended sample types, and consolidation of decision rules and supporting QA/QC for defined-scope confirmation.

Attribution-oriented status: not yet established.

5.4.5 Strychnine-type alkaloids

Identification readiness: R2

Confirmatory capability is under development. The analytical basis is strong and the compounds are well suited to LC–MS/MS, but readiness remains limited by matrix-specific testing, finalisation of scope, and integration into the broader cross-class workflow and reporting framework.

Attribution-oriented status: not yet established.

5.4.6 Trichothecenes

Identification readiness: R2

Confirmatory capability is under development. The principal challenge lies less in the existence of LC–MS methods than in matrix complexity, analyte diversity, and treatment of modified forms. Remaining

gaps relate to matrix-aware implementation, confirmatory logic for the intended scope, and practical integration of targeted and broader HRMS-based approaches.

Attribution-oriented status: not yet established.

5.4.7 Microcystins and nodularin

Identification readiness: R1–R2 depending on scope

Screening capability is established, and more selective confirmatory capability is emerging. However, readiness is still constrained by congener diversity, the distinction between free and total toxin, and the need to define how much orthogonal evidence is required for different matrix types. For this reason, the class is best regarded as screening-ready overall, with confirmatory capability under development for defined subsets.

Attribution-oriented status: not yet established.

5.5 Readiness summary table

Table 2. Readiness summary

Target / toxin class	Identification readiness	Attribution-oriented status	Main current gap
Botulinum neurotoxins	R1*	Preparatory only	Serotype-specific detection; consolidation of full evidential chain and confirmation scope
Ricin	**R3** (defined scope)	Preparatory only	Extension of robustness and explicit matrix/scope boundaries
PSTs / saxitoxin-group toxins	**R3** (defined scope)	Not yet established	Extension beyond best-characterised matrices and forensic scope expansion
Aconitum alkaloids	R2	Not yet established	Panel completion, matrix robustness, and confirmatory decision logic
Strychnine-type alkaloids	R2	Not yet established	Matrix robustness and integration into final decision/reporting framework
Trichothecenes	R2	Not yet established	Matrix complexity, modified forms, and final confirmatory scope
Microcystins and nodularin	R1–R2	Not yet established	Progression from broad screening toward clearly defined confirmatory scope

- BoNT/A capablility exists, work for other serotypes pending.

Overall, readiness in the first iteration can be described as **heterogeneous but strategically coherent**. The principal workflows for qualitative detection and identification are now defined, and

several targets have progressed beyond literature review into draft operational capability. At the same time, the readiness picture remains scope-bound, matrix-dependent, and clearly more advanced for identification than for attribution-oriented analysis. The first iteration should therefore be understood as having established the analytical foundation and direction of travel, while broader class expansion, stronger matrix coverage, and attribution-related development remain priorities for the second iteration.

6 IMPLICATIONS FOR ANALYTICAL ROP DEVELOPMENT

6.1 Identified prerequisites for future ROP development

The first iteration does more than identify a general need for Recommended Operating Procedures (ROPs). It also establishes a large part of the framework on which future ROPs will be built. In that sense, many of the prerequisites for later ROP development are no longer only prospective: they have already been defined at framework level in the current deliverable. The main task in subsequent iterations will therefore be to consolidate, refine, and scope these elements rather than to define them from first principles.

The current draft has already established several core prerequisites.

- **Defined analytical purpose and scope**
  The intended analytical use is now clearly framed as **qualitative detection and identification**, not routine quantification. The distinction between screening outputs and confirmatory identification has been defined, and the current target panel has been positioned as a first-iteration set of canonical exemplars rather than the final analytical scope. This provides an essential basis for future ROPs, because operating procedures cannot be written coherently unless the intended use and scope boundaries are explicit.
- **Defined workflow architecture**
  A common intake-and-routing concept has been established, together with the distinction between a low-molecular-weight toxin branch and a protein-toxin branch within one integrated analytical pathway. This is a major prerequisite for future ROP development, because it means that subsequent procedures can be built around a stable workflow logic rather than around isolated target-specific methods.
- **Defined evidential structure for confirmation**
  The current draft has also established the evidential logic required for decision-grade identification. In particular, it makes explicit that confirmatory readiness depends on completion of an evidential chain supported by orthogonal analytical dimensions rather than on the existence of a single screening assay. This is a central prerequisite for future ROPs, especially in a forensic or verification-oriented setting.
- **Defined QA/QC and decision-rule framework**
  The deliverable now provides a qualitative QA/QC framework based on predefined decision rules, acceptance criteria, and the use of decision threshold and detection capability concepts. This is important because future ROPs will need more than analytical steps: they will also need stable rules for when a result can be interpreted and reported.

- **Defined reporting framework**
  A reporting framework has been established for qualitative toxin identification, including the principle of controlled reporting categories, minimum reporting content, and explicit statement of limitations. Although this is not yet a final toxin-class-specific reporting code, it provides the necessary basis for future ROP-ready reporting templates.
- **Defined matrix-aware development principle**
  The present draft also makes clear that matrix diversity is one of the main determinants of readiness. Future ROPs will therefore need explicit matrix scope statements, sample-handling assumptions, and limitation language. This prerequisite is already recognised in the current document and does not need to be rediscovered in later work.
- **Defined supporting analytical assets**
  The current iteration has already generated draft workflow documents, preliminary analyte tables, peptide targets, reference spectra, qualitative identification criteria, and branch-specific analytical strategies. These materials do not yet constitute a full operational ROP package, but they are a necessary precursor to one.
- At the same time, some prerequisites remain only partly fulfilled and will require further work before mature ROPs can be claimed.
- **Technical consolidation within defined scope**
  Several workflows remain under development with respect to matrix robustness, analyte coverage, or completion of the full confirmatory evidence chain.
- **Within-laboratory reproducibility**
  Future ROPs will require more systematic demonstration that the qualitative outcome remains reproducible within the laboratory under expected variation in operator, day, and representative sample conditions.
- **Stable scope statements and limitation language**
  Although the need for explicit scope has been recognised, future ROPs will require more finalised statements on where a given procedure is valid and where analytical interpretation becomes limited.
- **Attribution-oriented analytical support**
  The current iteration has identified the kinds of analytical features that may become relevant for later attribution-oriented work, including impurity-related, purity-related, or variant-related observations. However, no attribution-ready workflow exists at present. This should not prevent development of first-generation detection and identification ROPs, but it does mean that attribution-oriented content should remain clearly separated from core identification procedures in the near term.

Overall, the main implication is that the present draft already provides much of the conceptual and structural groundwork required for later ROP development. Subsequent work should therefore concentrate on consolidation, scoping, and reproducibility rather than on redesign of the overall analytical framework.

6.2 Lessons learned for subsequent iterations

Several important lessons emerge from the first iteration and should guide the next phase of development.

- **A single universal procedure is not realistic**
  The first iteration confirms that one universal analytical method for all toxin classes and all matrices is neither practical nor analytically defensible. The more realistic solution is one integrated analytical pathway with a limited number of harmonised branches and clearly defined evidential routes.
- **Screening and confirmation must remain distinct**
  Rapid screening capability is valuable, but it is not equivalent to confirmatory readiness. The first iteration reinforces the need to keep screening/triage outputs and decision-grade identification conceptually separate. Future ROPs should preserve this distinction explicitly.
- **Progress depends on evidence-chain completion**
  The main developmental bottleneck is not necessarily lack of analytical signal generation, but incomplete evidential chains. Progress from early-stage capability to confirmation-ready status depends on whether orthogonal support, QA/QC, decision criteria, and reporting logic are all in place within the intended scope.
- **Matrix realism must remain central**
  Methods that are mature in narrow food, environmental, or laboratory matrices do not automatically translate into readiness for mixed, uncertain, or incident-related samples. Future iterations should continue to treat matrix scope and sample history as core determinants of readiness rather than as secondary complications.
- **Scope discipline is essential**
  The first iteration shows that readiness claims are only meaningful when tied to defined scope. Future ROPs should therefore remain explicit about intended matrices, evidential basis, and reporting boundaries, and should avoid broader claims than the data support.
- **The qualitative framework is appropriate and should be retained**
  The decision to frame the workflow as qualitative rather than quantitative has proved useful. It allows development to focus on the lower limit of defensible identification, evidential sufficiency, and reporting logic rather than on unrealistic requirements for uniform quantitative recovery across a broad toxin panel.
- **Attribution-oriented work should remain a separate development track**
  The first iteration also clarifies that attribution-oriented analysis should not be conflated with core detection and identification readiness. Preparatory analytical directions for later attribution work can already be identified, but substantive progress in this area will depend on access to suitable comparative materials and datasets. This is likely to remain a significant practical limitation in subsequent iterations.

These lessons point to a clear next-step strategy. The second iteration should focus on:

- moving currently incomplete targets toward defined-scope confirmatory readiness;
- strengthening matrix robustness and within-laboratory reproducibility;
- consolidating QA/QC, decision rules, and reporting logic into stable procedure packages;
- extending scope from the current canonical exemplars toward broader target coverage within the same toxin classes; and
- advancing attribution-oriented work cautiously, as a preparatory analytical line rather than as a prematurely claimed operational capability.

Taken together, the first iteration has established the analytical architecture, evidential principles, and reporting logic needed for future ROP development. The main challenge for subsequent

iterations is no longer to decide the general direction, but to convert this framework into stable, scope-defined, and transferable operating procedures.

7 KNOWN LIMITATIONS

This deliverable defines the validity boundary for the first iteration of EMBRACE T3.2. It establishes an integrated analytical and reporting framework, together with draft workflow elements and supporting annexes, but it does not constitute a universally transferable, fully validated, or accredited set of final operational ROPs. The limitations below are stated explicitly so that readiness claims, identification statements, and future ROP expectations are interpreted only within the scope documented in this deliverable and its annexes.

7.1 Scope and maturity limitations

First-iteration, framework-first scope. The current iteration prioritises the establishment of a limited number of harmonised analytical lanes, target-specific evidential logic, and qualitative reporting constructs. Several workflows have progressed to structured draft procedures, but they remain development frameworks pending further optimisation, performance evaluation, and procedural consolidation.

Heterogeneous maturity across targets and branches. Readiness remains uneven across the panel. The low-molecular-weight branch is currently the most harmonised, whereas the protein-toxin branch is more modular and more dependent on target-specific enrichment, functional evidence, and peptide-level confirmation. Across the panel, current status ranges from screening-ready to confirmation-ready within defined scope, with one target group remaining at not-initiated status for in-house experimental implementation.

Strategy/implementation distinction for some targets. For some targets, most notably botulinum neurotoxins, the analytical strategy and intended evidential chain are defined at framework level, but this should not be interpreted as current in-house confirmatory capability. Experimental implementation and readiness documentation for that target remain pending in this iteration.

Defined-scope validity. Any "confirmation-ready" claim is restricted to the target–matrix–evidential-chain combination for which decision rules, orthogonality, QA/QC elements, and reporting language are documented. Extension to new matrices, variants, or contexts requires explicit re-scoping and further verification.

7.2 Matrix limitations

Matrix effects and interferences. Complex matrices remain the dominant technical limitation across both major analytical branches and may affect selectivity, robustness, enrichment efficiency, ionisation behaviour, immunoreactivity, or functional readout.

Matrix-dependent transferability. A workflow demonstrated for one matrix category may not transfer directly to another without additional verification. This is particularly relevant for heterogeneous foods, environmental residues, wipe/swab extracts, and variable biofluids.
Surrogate-matrix basis of development. Much of the first-iteration development has been performed in surrogate matrices representing environmental, food, or biological sample types rather than authentic incident samples. These are necessary and informative for development, but they may not fully reproduce real-case behaviour.
Limited sample amount and heterogeneity. Restricted sample volume/mass, non-uniform materials, or difficult subsampling may constrain parallel progression through both analytical branches and reduce the extent of orthogonal follow-up achievable in a given case.

7.3 Reference materials, controls, and comparability limitations

Reference material availability. Access to appropriate reference materials, surrogates, and controls varies by target and can constrain verification breadth, sensitivity estimation, and confidence statements across matrices.
Reagent and control dependence. Some workflows, particularly for protein toxins, depend on suitable antibodies, enrichment reagents, and specialised controls, which are not equally available across targets and may constrain pace, scope, or transferability.
Single-laboratory implementation. WP3 is the only analytical laboratory in EMBRACE for this iteration. Results therefore reflect in-house development and verification rather than a multi-laboratory harmonisation dataset.
Limited inter-laboratory comparability evidence. Inter-laboratory comparison data and proficiency-testing support remain uneven across the panel and are not yet available in a form that supports broad cross-laboratory generalisation for all targets.
No claim of accreditation. While work is conducted according to quality principles consistent with ISO/IEC 17025-oriented practice, this deliverable does not constitute an accreditation claim, and reporting conventions are implemented within the project's present scope and maturity.

7.4 Reporting and performance-statement limitations

Qualitative-only reporting policy. EMBRACE outputs are qualitative ("identified" / "not identified", with additional limited categories where required) and are supported by documented decision rules and orthogonal evidence. Routine quantitative claims are outside the intent of this iteration.
Non-identification is conditional, not absolute. A result reported as "not identified" means that the toxin was not identified under the applied workflow, matrix conditions, and decision rule; it should not be interpreted as proof of absolute absence.

Confidence statements are evidence-based and scoped. Identification statements are limited to the evidential basis documented for each target and branch. Results cannot be generalised beyond the stated evidential chain, matrix scope, and QA/QC-supported interpretation.

Draft templates and annexes remain evolving. Reporting templates, annexes, and protocol-style workflow texts will continue to be refined as additional verification data become available. Current texts should therefore be understood as first-iteration development assets rather than finalised, stable ROPs.

No standalone attribution claims in this iteration. Although the broader task includes provenance-support ambitions, this deliverable does not provide an internal attribution dataset or a basis for standalone attribution claims. Any future impurity- or proteoform-based support will require dedicated development, validation, and explicit scoping.

7.5 Practical constraints affecting delivery

Resource and scheduling dependencies. Development pace can be affected by instrument availability, competing operational priorities, and the time required to obtain suitable materials, controls, and specialist reagents.

Not all targets progressed experimentally. Some targets remain at screening-ready or confirmation-under-development status, and botulinum neurotoxin work has not yet started in-house at the experimental level in the current iteration.

Consolidation still required before ROP release. Several workflow elements still require analyte-specific optimisation, matrix-specific performance evaluation, and consolidation of controls, acceptance logic, and reporting language before they can be stabilised as ROPs for defined scope.

8 ROADMAP TOWARDS 2ND ITERATION

The second iteration should be organised around a clear practical principle: **analytical development should be substantially completed by Month 30**, leaving the final six months for consolidation, drafting of formal ROPs, internal review, partner feedback, and completion of the final deliverable. This is important because the remaining work is no longer primarily strategic. The main analytical architecture has already been defined in the current iteration, and the next phase should therefore focus on converting that framework into defined-scope, reproducible, and reportable operating procedures.

The roadmap should also reflect the role of the current target panel. The toxins addressed in the first iteration should continue to be treated as **canonical exemplars** of broader toxin classes. The critical path for the second iteration is therefore not unlimited scope expansion, but completion and consolidation of the evidential chains for the current representative targets. Broader class expansion should be pursued only where it can be achieved without delaying the completion of the first ROP package.

8.1 Planned analytical developments

The second iteration should prioritise six linked development streams.

8.1.1 Completion of confirmatory workflows for first-iteration targets

The main analytical priority is to move targets that are currently screening-ready or confirmation-under-development towards defined-scope confirmatory readiness. In practice, this means consolidating the evidential chain for the current representative targets, especially where the analytical route is already defined but matrix scope, decision rules, or QA/QC logic remain incomplete.

8.1.2 Matrix-focused strengthening of workflow robustness

A major part of the remaining work should be matrix-focused rather than analyte-focused. The objective is to verify that the chosen workflows remain interpretable across the priority matrix categories already defined in the current iteration, including representative environmental, food, and biological sample types. This should be treated as a prerequisite for credible defined-scope ROPs.

8.1.3 Consolidation of QA/QC, decision rules, and reporting logic

The next phase should convert the current qualitative framework into stable workflow-specific packages. This includes branch-specific QA/QC structures, acceptance criteria, lower limits of qualitative detection or detection capability estimates, and fixed reporting categories. By the end of the analytical phase, these elements should no longer remain draft concepts but should be integrated into the working procedures.

8.1.4 Completion of supporting analytical assets

The reference spectra, analyte tables, peptide targets, diagnostic fragments, and supporting annexes generated in the current iteration should be completed and stabilised for the targets retained in the defined scope. These assets will form the technical backbone of the future ROPs and should therefore be treated as part of the critical path rather than as optional supporting material.

8.1.5 Limited extension of target scope within the same toxin classes

Where standards, reagents, and development time permit, the second iteration should expand beyond the canonical first-iteration exemplars to additional targets within the same toxin classes. However, such expansion should remain secondary to completion of the core evidence chains for the current panel.

8.1.6 Attribution-oriented analytical work

Attribution-oriented analysis should be implemented as a defined second-iteration development stream and included in the second deliverable. The current iteration has already identified the principal analytical dimensions that may support later attribution-oriented interpretation, including impurity-related observations, purity-linked features, variant or proteoform discrimination, and other comparative analytical characteristics beyond simple identification. In the second iteration, this line of

work should be developed in a bounded and target-prioritised manner, with emphasis on those targets for which comparative materials and analytically meaningful distinguishing features can realistically be obtained. Because access to multiple relevant preparations is likely to be limited, the expected output should be framed as an initial attribution-support package — for example, candidate analytical features, comparison logic, and defined limitations — rather than as a fully mature provenance workflow for all targets.

8.2 Expected timeline for introducing ROPs

The remaining project period extends from Month 18 to Month 36. A practical and realistic timeline is outlined below.

Table 3. Implementation timeline for analytical workflow maturation and ROP development

Period	Main focus	Expected output
M18–M21	Scope freeze and attribution planning	Final prioritisation of first-iteration targets and matrices; identification of critical missing reagents, standards, and comparative materials; identification of candidate analytical features relevant to attribution (e.g. impurity profiles, variant distributions)
M21–M24	Targeted analytical development	Focused experimental work on targets still below confirmation-ready level; strengthening of matrix-specific workflows; extension of confirmatory chains where key modules already exist
M24–M27	Consolidation and reproducibility	Within-laboratory reproducibility checks; refinement of QA/QC, decision rules, and reporting logic; completion of supporting analytical assets
M27–M30	Freeze of analytical content	Finalisation of the defined-scope workflow package for first-generation ROPs; updated readiness assessment; locking of core analytical procedures, limitations, and supporting annex material; preliminary evaluation of attribution-relevant analytical features
M30–M33	ROP drafting and internal review	Drafting of formal ROP texts and associated reporting templates; internal consistency review across branches and targets
M33–M36	Revision and deliverable completion	Partner review, revision, integration into final deliverable, and final editorial and quality-assurance checks

This schedule deliberately front-loads analytical work. The intention is that **by Month 30 the analytical content is substantially complete**, so that the final six months can be devoted primarily to formalisation, review, and deliverable production rather than to unfinished core method development.

A practical implication of this timeline is that not every desirable development can be pursued at equal depth in parallel. If trade-offs become necessary, first priority should remain completion of confirmatory readiness for the current canonical targets and consolidation of QA/QC and reporting logic. However, attribution-oriented analysis must also be progressed during the second iteration as a defined deliverable component. In practical terms, this means that attribution work should be scoped selectively and tied to targets and analytical features for which meaningful comparative evaluation is feasible within the project period.

8.3 Interfaces with T3.1 and T3.3

The roadmap for the second iteration should remain closely linked to the wider Work Package 3 context.

8.3.1 Interface with T3.1

The Description of Action already links the analytical work in Task 3.2 to the matrix categories and use contexts defined in Task 3.1, including environmental samples, powders, food extracts, and biological fluids. In practical terms, this means that T3.1 should continue to inform which matrices, sample histories, and incident-use assumptions are prioritised in the second iteration. The analytical work should not evolve independently of these operational assumptions. Conversely, the limitations identified in Task 3.2 should feed back into T3.1 so that sampling and evidential expectations remain realistic.

8.3.2 Interface with T3.3

The current draft also states that Task 3.3 addresses field-deployable biodetection technologies, while Task 3.2 provides the laboratory-based confirmatory capability needed after field screening. This interface should be strengthened in the second iteration. In practical terms, T3.3 should inform which preliminary indicators or field outputs are most likely to require laboratory follow-up, while T3.2 should define how such samples enter the confirmatory workflow and how the evidential status of field-derived indications is translated into laboratory reporting.

8.3.3 Practical coordination need

For the second iteration, these interfaces should be treated as active coordination points rather than background assumptions. At minimum, coordination should ensure that:

- matrix priorities remain aligned with scenario needs;
- field-screening outputs and laboratory confirmation outputs are not described in contradictory terms;
- and reporting language remains consistent across the broader WP3 chain of evidence.

8.4 Overall roadmap conclusion

The second iteration should be organised around a simple principle: **complete first-generation analytical workflows early, then formalise them**. The main analytical work should therefore be

concentrated between **Month 18 and Month 30**, with the remaining period used for ROP drafting, internal consolidation, partner review, and final deliverable preparation.

If this schedule is maintained, the second iteration should deliver not only defined-scope confirmatory workflows for the current canonical targets, but also an initial attribution-support component identifying the comparative analytical features, feasible comparison approaches, and principal limitations relevant to provenance-oriented interpretation within the EMBRACE framework.

9 CONCLUSIONS

9.1 Summary of analytical insights

The first iteration indicates that a robust EMBRACE analytical capability for biotoxins is most credibly developed through (i) a limited number of harmonised analytical lanes, (ii) strict separation of screening and confirmatory outputs, and (iii) a transparent qualitative reporting framework anchored in orthogonal evidence and documented decision rules. The work further shows that matrix diversity is the dominant practical constraint across the toxin panel and that method development is most usefully approached as completion of evidential chains within a defined matrix scope, rather than as isolated technical optimisation.

The readiness assessment provides a realistic picture of current capability across the panel. For some targets, confirmatory capability is already achievable within a defined scope, whereas others remain at screening-ready or confirmation-under-development stage, and one target group has not yet been experimentally progressed in this iteration. Explicit definition of scope, limitations, and uncertainty is therefore not supplementary to the EMBRACE approach, but a core condition for defensible analytical outputs.

9.2 Contribution to EMBRACE objectives

This deliverable contributes to EMBRACE by establishing a structured and quality-oriented analytical framework for biotoxin detection and identification that is aligned with the project's civil and defensive purpose and suitable for incident-relevant interpretation. In particular, it:

- translates the EMBRACE target toxin panel into implementable analytical and reporting constructs suitable for staged development into ROPs;
- establishes consistent terminology and decision logic distinguishing screening from confirmation, thereby reducing the risk of over-interpretation of preliminary indications;
- embeds orthogonality, transparency, and defined-scope reporting as project-level principles for qualitative toxin identification;
- provides a readiness-based development framework that supports prioritisation of second-iteration work, including confirmatory workflow consolidation, attribution-supporting analytical development, and continued integration with sampling and traceability work in T3.1 and field-facing capability development in T3.3.

Overall, the first iteration provides the analytical foundation for the second iteration, in which scope, robustness, and reporting maturity will be extended through target-prioritised method development, staged ROP drafting, and inclusion of an initial attribution-support component within the defined EMBRACE framework.

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ANNEXES

Annex A – In-depth overview of target biotoxin families

Annex B – Analytical Readiness Level Framework

Annex C – Summary of Analytical Evidence

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A ANNEX A - BACKGROUND INFORMATION ON TARGET BIOTOXIN FAMILIES

Purpose of this annex

This annex provides concise toxicological background on the biotoxin families addressed in the EMBRACE analytical framework. Its purpose is to summarise the biological origin, structural characteristics, mechanisms of action, key toxicological properties, and, where relevant, selected analytical implications of each toxin group in order to support interpretation of the findings presented in the main report.

The annex is intended as contextual reference material complementing the analytical discussion in Sections 3.2 and 4. It is not intended to provide full analytical protocols or validation data, but it may include concise comments on analytical relevance, evidential interpretation, and matrix-specific considerations where these are important for understanding the significance of a toxin class in environmental, public-health, forensic, and security-related scenarios.

A.1 Ricin

Ricin is a plant-derived, heterodimeric type II ribosome-inactivating protein (RIP-II) and is specifically listed under Schedule 1 of the Chemical Weapons Convention Annex on Chemicals (CAS 9009-86-3) (Organisation for the Prohibition of Chemical Weapons, OPCW).

Attribute	Description
Substance	Ricin
CAS	9009-86-3 (OPCW)
Protein class	Type II ribosome-inactivating protein (RIP-II), A–B toxin (Audi et al., 2005)
Architecture	Disulfide-linked A chain (catalytic) + B chain (lectin, cell-binding) (Audi et al., 2005)
Approx. size	~60–65 kDa glycoprotein (mass varies with glycosylation/proteoforms) (Audi et al., 2005; Worbs et al., 2015)
Stability (high-level)	Stable under normal conditions; loss of activity with sufficiently high heat (context-dependent) (Centers for Disease Control and Prevention, 2024)

A.1.1 Purpose and regulatory context

Ricin is one of only two biotoxins explicitly named in CWC Schedule 1 (the other commonly cited example is saxitoxin), which makes it unusually prominent from a verification and analytical-preparedness perspective compared with most naturally occurring toxins (OPCW, n.d.).

Beyond the CWC framework, ricin is regulated in other national schemes; for example, it appears on the HHS and USDA Select Agents and Toxins list maintained by the Federal Select Agent Program (Federal Select Agent Program, 2025).

Ricin in real samples is often not a single molecular entity: it is frequently a mixture of isolectins and proteoforms whose sequence similarity to *Ricinus communis* agglutinin (RCA120), combined with variable glycosylation and deamidation, can confound screening assays and complicate confirmatory

peptide calling unless workflows are designed with that heterogeneity in mind (Bergström et al., 2015; Josuran et al., 2024; Worbs et al., 2015).

A.1.2 Biological origin and gene-level diversity

Ricin is produced by the castor bean plant and accumulates in seeds; at the biosynthetic level it is made as a single precursor polypeptide (preproricin/proricin) that contains both A-chain and B-chain sequences and is routed through the secretory system before being processed into the mature disulfide-linked heterodimer (Lord & Spooner, 2011).

A key background point for "genes and variance" is that the castor bean genome contains a large ricin/RCA gene family, not a single ricin gene. In the published genome analysis, 28 family members were identified, and domain analysis indicated that 7 genes encode full-length proteins containing the RIP domain plus two lectin domains (i.e., ricin-like A–B architectures), while other family members encode partial domain architectures (RIP-only and/or lectin-only) (Chan et al., 2010).

Ricin itself is commonly discussed as two principal isolectins (ricin D and ricin E) alongside the related lectin RCA120 (agglutinin) (Araki & Funatsu, 1987; Worbs et al., 2015). Importantly, ricin E is not merely a minor point-mutation variant; classic protein sequencing work showed the B chain of ricin E to be a recombination product, composed of an N-terminal portion matching ricin D and a C-terminal portion matching RCA120, consistent with gene recombination between the respective B-chain genes (Araki & Funatsu, 1987).

Cultivar and provenance contribute additional, practically relevant variance. Analytical studies comparing ricin preparations from multiple horticultural varieties and geographic origins have demonstrated extensive heterogeneity consistent with expression from a multigene family and superimposed post-translational variation (Despeyroux et al., 2000; Josuran et al., 2024).

A.1.3 Protein structure and post-translational heterogeneity

Ricin is an A–B toxin. The B chain is a galactose/GalNAc-binding lectin with two major domains, each harbouring a carbohydrate-binding site, while the A chain is the catalytic subunit that ultimately inactivates ribosomes (Rutenber & Robertus, 1991; Spooner & Lord, 2015).

A.1.4 N-glycosylation sites and glycoform diversity

Ricin is a glycoprotein, and a major driver of ricin "isoform complexity" is N-glycosylation. Contemporary characterisation work agrees that ricin contains four N-glycosylation motifs that are typically glycosylated—two on the A chain and two on the B chain—and that glycosylation contributes materially to the heterogeneity observed across preparations and cultivars (Josuran et al., 2024; Worbs et al., 2015). Certified reference material work on ricin CRM-LS-1 reports glycosylation sites at A:N10, A:N236, B:N95, and B:N135 and provides a detailed plant-type N-glycan profile that includes multiple oligomannosidic and paucimannosidic structures together with site-specific occupancy distributions (Josuran et al., 2024).

A.1.5 Sequence variants, isolectins, and deamidation-driven microheterogeneity

Isolectin differences matter analytically. In characterised material, ricin D and ricin E share an identical A-chain sequence, while differences are concentrated in the B chain, consistent with the recombination history described above. This affects peptide selection for confirmatory MS because

some B-chain peptides can be isolectin-specific, whereas A-chain peptides are more conserved across D and E (Araki & Funatsu, 1987; Worbs et al., 2015).

A further layer of complexity is deamidation (Asn→Asp/isoAsp), which can generate charge variants and contribute to the isoelectric diversity observed in ricin preparations. A dedicated CZE- and LC-MS study confirmed deamidation at three asparagine residues (one in the A chain and two in the B chain) in the preparation studied and linked this to observed heterogeneity (Bergström et al., 2015).

Finally, there is evidence that ricin isoforms can differ in biological potency and that glycosylation level correlates with differential toxicity profiles across isoforms in experimental studies, reinforcing why proteoform-level characterisation is not merely academic for risk interpretation and reference material choice (Sehgal et al., 2011).

A.1.6 Cellular mechanism and pathophysiology

Ricin toxicity begins with lectin-mediated binding of the B chain to galactose- and N-acetylgalactosamine-containing glycoconjugates on mammalian cell surfaces; this broad receptor availability is one reason ricin can affect many tissues and present with non-specific systemic syndromes (Spooner & Lord, 2015).

After endocytosis, a small fraction of internalised ricin traffics via retrograde pathways to the trans-Golgi network and endoplasmic reticulum. In the ER, the A chain is released and ultimately reaches the cytosol through processes that intersect with host quality-control machinery, including ER-to-cytosol dislocation routes (Lord & Spooner, 2011; Spooner & Lord, 2015).

Once cytosolic, the A chain acts as an RNA N-glycosidase, depurinating a single conserved adenine residue in the sarcin–ricin loop of 28S rRNA (classically reported as A4324 in rat 28S rRNA), thereby preventing key elongation factors from interacting with the ribosome and arresting protein synthesis (Endo & Tsurugi, 1988).

Ricin's intracellular itinerary is not passive. Mechanistic work supports that features such as low lysine content in toxins that transit from the ER to the cytosol can reduce susceptibility to ubiquitin-mediated degradation, contributing to successful intoxication despite cellular degradation pathways (Deeks et al., 2002; Spooner & Lord, 2015).

Downstream pathophysiology extends beyond translation arrest to include inflammatory signalling and tissue injury cascades, including acute inflammatory responses and severe organ injury in experimental systems (Korcheva et al., 2005; Wilhelmsen & Pitt, 1996).

A.1.7 Toxicokinetics and route-dependent clinical picture

A consistent cross-source conclusion is that ricin risk is strongly route-dependent: ingestion typically produces predominantly gastrointestinal injury, whereas inhalation and parenteral or injection exposures are associated with much more severe systemic outcomes and higher lethality in documented scenarios and animal models (Audi et al., 2005; Worbs et al., 2011).

Ingestion: Oral exposure is usually characterised by nausea, vomiting, abdominal pain, and diarrhoea; severity depends on factors such as mastication, which increases toxin release, and the timing of supportive care. Large case reviews emphasise that, with treatment, many patients recover, and that

mortality assumptions based purely on animal extrapolation can overstate risk for typical seed ingestions under modern care (Audi et al., 2005; Worbs et al., 2011).

Inhalation: Inhalational exposure is the route most consistently associated with severe pulmonary toxicity in animal models, including acute respiratory distress, marked thoracic pathology, and progression over hours to days. Non-human primate pathology studies report lesions largely confined to the thoracic cavity following lethal inhalational challenge, supporting the view that the respiratory tract is the primary target organ for aerosols (Wilhelmsen & Pitt, 1996). Experimental work also shows that exposure modality and particle-size distribution influence respiratory deposition and delivered dose in small-animal models, which is analytically and clinically relevant when interpreting aerosolised exposure studies or designing realistic sampling strategies (Roy et al., 2003).

Parenteral exposure and historical case context: clinical compilations indicate that intended poisonings involving parenteral routes have markedly higher fatality than accidental ingestions. A large review of real cases reported a fatality rate of around 1.5% for accidental human intoxications considered, predominantly ingestion, whereas intended poisonings showed much higher mortality in the compiled dataset (Worbs et al., 2011). The most widely cited historical assassination case is that of Georgi Markov in London in 1978, which remains a canonical reference point in forensic discussions of ricin because it illustrates the high lethality potential of parenteral delivery even when overall population-level dissemination scenarios are less plausible (Crompton & Gall, 1980).

Dermal and ocular contact: Multiple reviews note that dermal absorption through intact skin is unlikely or unproven, and that the highest-risk exposure routes remain inhalation and injection. This is important when framing "contact" scenarios and when prioritising sampling matrices such as skin swabs versus aerosols versus ingested materials (Centers for Disease Control and Prevention, 2024; Schep et al., 2009).

A.1.8 Clinical management and countermeasure status

Authoritative clinical reviews and public health sources emphasise that management is primarily supportive—airway and respiratory support, fluids and electrolytes, and symptom-directed care—and that there are no widely available licensed specific antidotes for ricin intoxication (Audi et al., 2005; Centers for Disease Control and Prevention, 2024).

Countermeasure development is active. Recombinant A-chain-based vaccine candidates such as RiVax and RVEc programmes have progressed through early clinical development, and monoclonal antibodies with activity against both D and E isoforms show strong protection in animal models, supporting the direction of travel even though these are not yet routine clinical deployments (Vance & Mantis, 2016).

A route-syndrome summary table is below.

Exposure route	Primary target physiology	Typical clinical pattern (high level)	Severity tendency	Practical diagnostic implication
Ingestion	GI mucosa	Gastroenteritis with dehydration; systemic collapse uncommon in	Often survivable with supportive care, but can be severe	Direct toxin detection can be difficult; history plus symptom pattern important

		many seed ingestions with care		
Inhalation	Respiratory tract	Fever/cough followed by progressive respiratory distress; pulmonary injury dominates	High concern in animal models; potentially fatal	Respiratory samples and environmental/aerosol sampling are high priority
Injection/ parenteral	Systemic	Rapid systemic toxicity and higher fatality in documented intended cases	Highest lethality in case compilations	Traceability and forensics are critical; exposure-route inference affects prognosis
Dermal/ocular	Surface contact	Local irritation possible; systemic intoxication via intact skin not demonstrated in key reviews	Lower, unless assisted by wounds or other factors	Swab sampling may be useful for attribution even if systemic risk is limited

This table synthesises evidence and consensus statements from clinical reviews and case compilations (Audi et al., 2005; Centers for Disease Control and Prevention, 2024; Worbs et al., 2011).

A.1.9 Analytical implications and recommended evidence chain for EMBRACE

Analytical challenges in real samples. Ricin analysis is complicated by three overlapping realities: (i) co-expressed isolectins, especially D and E; (ii) the presence of RCA120 and other family members with high sequence similarity; and (iii) substantial glycosylation and deamidation heterogeneity that generates multiple proteoforms and charge variants (Bergström et al., 2015; Josuran et al., 2024; Worbs et al., 2015).

For EMBRACE panel design, this argues for orthogonal confirmation rather than single-assay reliance, especially when results may be used for high-consequence decisions. Performance in inter-laboratory exercises shows that either sophisticated MS approaches or combinations of immunological, functional, and MS strategies can successfully identify ricin across complex matrices such as buffer, milk, meat extract, and fertiliser-like samples (Kalb et al., 2015; Worbs, Skiba, Bender, et al., 2015).

Screening methods. Immunoassays remain the most common rapid screening tools. Proficiency-test analyses describe multiple workable immunological strategies, including ELISA variants and immunochromatographic or lateral-flow formats, but also highlight the recurring need to differentiate ricin from RCA120 explicitly (Simon et al., 2015; Worbs, Skiba, Söderström, et al., 2015).

Independent evaluations of commercial field detection assays show variable performance around manufacturer-claimed detection limits, reinforcing that on-site positives should be treated as presumptive until confirmatory testing is completed (Slotved et al., 2014).

Confirmatory identification and isoform-aware proteomics. Mass spectrometry enables confirmation via protein sequence evidence, especially signature peptides, and is particularly powerful for distinguishing ricin from RCA120 when peptides are chosen to target discriminating regions. Multiple peer-reviewed workflows demonstrate immunocapture or enrichment followed by MALDI-

TOF or LC-MS/MS peptide mapping for ricin detection in complex samples (Duriez et al., 2008; Kalb et al., 2015). Because ricin E differs from ricin D mainly in the B chain, an isoform-aware confirmatory strategy benefits from (a) A-chain peptides for broad ricin detection and (b) selected B-chain peptides for D/E discrimination and for exclusion of RCA120 where ambiguity exists (Josuran et al., 2024; Worbs, Skiba, Söderström, et al., 2015).

Activity-aware confirmation. Ricin risk is driven by biological activity, not just immunoreactivity. MS-based approaches can monitor ricin enzymatic activity by measuring adenine release from depurinated substrates, while cell-based assays provide a functional readout of cytotoxic activity, with published sensitivity ranges and matrix effects noted in proficiency-test discussions (Kalb et al., 2015; Worbs, Skiba, Bender, et al., 2015). Reference material work using highly purified ricin and RCA120 supports functional differentiation: ricin shows substantially higher cytotoxicity than RCA120 in real-time cytotoxicity assays, which is helpful when interpreting activity assays in the presence of homologous lectins (Worbs et al., 2015).

Biomarkers for exposure attribution. Direct detection of ricin in human biofluids can be challenging because the toxin is rapidly taken up into tissues; therefore, small-molecule markers associated with castor products can support attribution. **Ricinine** has been validated as a urinary biomarker of exposure to castor bean products and has also been studied in population baselining work to characterise background levels (Hamelin et al., 2012; Pittman et al., 2013).

Reference materials and the importance of documenting glycoforms. Certified and well-characterised reference materials matter because ricin is not a single uniform analyte. Detailed glycan profiling of ricin certified reference material CRM-LS-1 demonstrates that glycosylation is a defining characteristic of the material and varies across origins and isoforms; that variability has direct implications for assay calibration, targeted peptide choice, and inter-laboratory comparability (Josuran et al., 2024; Worbs, Skiba, Söderström, et al., 2015).

A.2 Botulinum neurotoxins

Botulinum neurotoxins (BoNTs) are exceptionally potent bacterial protein neurotoxins produced primarily by anaerobic clostridia and responsible for botulism, a severe neuroparalytic syndrome characterised by acute, symmetric, descending flaccid paralysis with prominent cranial nerve (bulbar) involvement and risk of respiratory failure (CDC, 2021; Arnon et al., 2001).

BoNTs are *not* a single analyte: they form a genetically and structurally diverse family. Classical clinical/public-health frameworks recognise **seven serotypes (A–G)**, defined serologically by neutralisation, yet many subtypes (>40) and mosaic toxins exist, and additional divergent BoNT/BoNT-like molecules have been reported in clostridial and non-clostridial hosts (Hill et al., 2007; Peck et al., 2017; Zhang et al., 2017; Zhang et al., 2018; Zornetta et al., 2016). Human botulism is caused predominantly by serotypes A, B, E, and F, whereas serotypes C and D are more strongly associated with animal disease (Arnon et al., 2001; Montal, 2010; Smith et al., 2005; Peck et al., 2017; Rummel, 2013).

Mechanistically, BoNTs are modular AB toxins: a ~150 kDa holotoxin comprising a zinc-dependent protease light chain (LC; ~50 kDa) linked by disulfide bond to a heavy chain (HC; ~100 kDa) that mediates binding to neuronal receptors and translocation of LC into the cytosol (Lacy et al., 1998; Montal, 2010). Their LC proteases cleave SNARE proteins (e.g., SNAP-25, VAMP/synaptobrevin, syntaxin), disabling synaptic vesicle fusion and thus blocking acetylcholine release at peripheral cholinergic nerve terminals (Blasi et al., 1993a; Schiavo et al., 1992; Montecucco & Schiavo, 1994).

From an analytical/forensic perspective, BoNT investigations benefit from a layered evidential approach: (i) functional activity confirmation (e.g., Endopep-MS), (ii) structural identification and serotype/subtype discrimination by proteomics, and (iii) organism/genomic context *only when organism/spores are present* (Boyer et al., 2005; Kalb et al., 2015; Dorner et al., 2013; Lindström & Korkeala, 2006).

BoNTs are a family of bacterial neurotoxins that evolved to target synaptic transmission. In nature and in many exposure contexts, BoNT holotoxin is packaged with non-toxic neurotoxin-associated proteins (NAPs) into progenitor toxin complexes (PTCs) that increase environmental persistence and facilitate gastrointestinal survival and uptake, which is central to foodborne botulism risk (Gu et al., 2012; Lee et al., 2013).

A.2.1 BoNT identity and molecular architecture

BoNTs are bacterial protein neurotoxins produced mainly by clostridia (especially *Clostridium botulinum*), but toxin genes and/or toxin production also occur in other clostridia such as *C. baratii* (type F) and *C. butyricum* (type E), which is clinically relevant because toxin identity and bacterial host do not always align one-to-one (Hall et al., 1985; Aureli et al., 1986).

Classically, BoNTs are grouped into **serotypes A–G** based on lack of cross-neutralisation by serotype-specific antisera (Arnon et al., 2001; Lindström & Korkeala, 2006). Beyond this framework, additional BoNT and BoNT-like molecules have been reported, including BoNT/X (Zhang et al., 2017), BoNT/En in *Enterococcus faecium* (Zhang et al., 2018), and BoNT/Wo in *Weissella oryzae* (Zornetta et al., 2016); and the "type H" report is now generally treated as a mosaic context, with nomenclature and classification discussed in the literature (Dover et al., 2014; Maslanka et al., 2016; Peck et al., 2017).

At the protein level, the **BoNT holotoxin** is ~150 kDa, composed of a ~50 kDa LC (a zinc metalloprotease) and a ~100 kDa HC linked by a single interchain disulfide bond (Lacy et al., 1998; Montal, 2010). The HC comprises an N-terminal translocation domain (HN) and a C-terminal receptor-binding domain (HC in domain nomenclature), giving the canonical LC–HN–HC modular organisation (Lacy et al., 1998; Montal, 2010).

Structural biology has established representative folds and domain organisation for multiple serotypes. For example, the crystal structure of BoNT/A (PDB: 3BTA) provided early, high-impact evidence for the modular architecture and implications for toxicity mechanisms (Lacy et al., 1998). The structure of BoNT/B (PDB: 1F31) describes catalytic and binding-site features in a serotype that uses synaptotagmin receptors, supporting mechanistic understanding of receptor recognition and toxicity (Swaminathan & Eswaramoorthy, 2000).

In natural material, BoNT is usually not present as a free 150 kDa holotoxin but as part of a **progenitor toxin complex (PTC) assembled with neurotoxin-associated proteins (NAPs)**. The core NAP is NTNHA (non-toxic non-haemagglutinin), which binds BoNT tightly to form the minimal PTC (M-PTC); structural work showed that this is an interlocked BoNT–NTNHA heterodimer in which NTNHA shields the toxin from low pH and proteolysis during gastrointestinal transit (Gu et al., 2012). In HA-positive clusters, additional NAPs—HA70, HA17, and HA33—associate with the M-PTC to form the large PTC (L-PTC); for serotype A, Lee et al. described an approximately 760 kDa, 14-subunit complex in which the BoNT–NTNHA module provides protection, whereas the HA module contributes to intestinal attachment and absorption by engaging host glycans (Lee et al., 2013). By contrast, OrfX-type toxin clusters encode OrfX1–3 and P47 instead of HA proteins; these proteins are now recognized as alternative NAP components, although their structural and functional roles have historically been less well defined than those of the HA complex (Gustafsson et al., 2017). Together, these protein assemblies explain why oral botulism depends not only on the catalytic neurotoxin itself, but also on accessory proteins that stabilize, package, and deliver it in the gut environment (Gu et al., 2012; Lee et al., 2013).

Genetically, *bont* genes are typically embedded within a neurotoxin gene locus, commonly including *ntnh* adjacent to *bont,* and accessory genes whose architecture varies in ways relevant to biology, detection, and attribution (Lúquez et al., 2009; Brunt et al., 2018). Two major organisational patterns are widely discussed: ha-cluster arrangements, including ha genes (e.g., HA70/HA33/HA17) and the alternative sigma factor regulator botR, versus orfX-cluster arrangements containing *orfX* genes (often with p47/p21-type elements) (Lúquez et al., 2009; Brunt et al., 2018).

Expression is regulated rather than purely constitutive. For instance, BotR/A acts as a positive regulator for BoNT/A complex genes in *C. botulinum* (Marvaud et al., 1998), and CodY can positively regulate botA expression in relation to nutritional state (Zhang et al., 2014). These regulatory features can influence toxin yields and thereby affect risk assessment and interpretation of culture-based findings (Marvaud et al., 1998; Zhang et al., 2014).

Finally, the physical genomic location of *bont* can vary. BoNT-encoding genes have been demonstrated on large plasmids in some strains (Marshall et al., 2007), and experimental work has

shown conjugative transfer of BoNT-encoding plasmids between *C. botulinum* strains (Marshall et al., 2010). In type C/D systems, classic experimental work showed prophage curing and conversion of toxigenicity linked to specific phages, consistent with phage-mediated mobility for certain toxin systems (Eklund & Poysky, 1974). This mobility underpins why serotype alone is an incomplete descriptor for source attribution and why genomic context can matter when available (Marshall et al., 2007; Marshall et al., 2010; Eklund & Poysky, 1974; Hill et al., 2007).

A.2.2 Biological origin and environmental occurrence

BoNT production is distributed across multiple clostridial lineages rather than confined to a single, uniform species concept; this is one reason BoNT toxicology and forensic interpretation often need to treat "toxin" and "organism" as related but separable entities (Lindström & Korkeala, 2006; Hill et al., 2007).

Within *C. botulinum*, strains are often described in broad physiological/genomic groups (I–IV) that differ in traits such as proteolysis, growth temperature range, and ecological associations; groups I and II are the principal sources of human botulism-associated toxin types, while group III is commonly associated with animal botulism and group IV is less often associated with human disease (Lindström & Korkeala, 2006). These groupings matter for *toxin sources* (i.e., where BoNT may originate environmentally) more than for laboratory identification per se in this dossier (Lindström & Korkeala, 2006; Hill et al., 2007).

The ecological reservoir includes spores that persist in soils and sediments; spore ecology supports long-term environmental presence and episodic contamination of foods or wounds, which underlies natural exposure scenarios (Lindström & Korkeala, 2006; World Health Organization, n.d.).

Beyond *C. botulinum*, toxin production by other clostridia has been documented in human disease contexts—e.g., type F toxin production linked to an organism resembling *C. baratii* in infant botulism (Hall et al., 1985), and type E infant botulism linked to neurotoxigenic *C. butyricum* (Aureli et al., 1986). These findings demonstrate clinically relevant toxin production outside classical *C. botulinum* framing and reinforce that organism-centred assays (culture/PCR) and toxin-centred assays (activity/proteomics) answer different questions (Hall et al., 1985; Aureli et al., 1986; Lindström & Korkeala, 2006).

Genomic surveys and functional characterisation have broadened the evolutionary space: a BoNT-like toxin in *E. faecium* (BoNT/En) cleaves SNARE proteins at sites distinct from canonical serotypes, illustrating that BoNT-like functions are not exclusive to clostridia (Zhang et al., 2018; Brunt et al., 2018). Likewise, a non-clostridial BoNT-like metalloprotease from *W. oryzae* (BoNT/Wo) cleaves VAMP at a novel, functionally relevant site (Zornetta et al., 2016). These findings expand the plausible diversity that broad-coverage detection systems must consider (Zhang et al., 2018; Brunt et al., 2018; Zornetta et al., 2016).

A.2.3 Mechanism of action

BoNT intoxication proceeds through conserved steps: (i) binding to neuronal surface glycans and protein receptors, (ii) endocytosis into synaptic vesicles/endosomes, (iii) endosomal acidification triggering HN-mediated translocation of LC into the cytosol, and (iv) LC proteolysis of specific SNARE proteins needed for synaptic vesicle fusion (Montecucco & Schiavo, 1994; Montal, 2010).

Receptor recognition is a major determinant of neurospecificity. BoNTs typically bind gangliosides as low-affinity co-receptors and then engage a protein receptor that confers high-affinity uptake into nerve terminals (Montecucco & Schiavo, 1994; Lam & Jin, 2015). For BoNT/A, synaptic vesicle glycoprotein 2 (SV2) isoforms serve as protein receptors mediating entry (Dong et al., 2006). For BoNT/B, synaptotagmin I/II are key protein receptors, and double receptor anchorage helps explain high specificity (Rummel et al., 2007; Dong et al., 2003).

After endocytosis, acidification drives conformational changes enabling the HN domain to translocate LC across the vesicular membrane; reduction of the interchain disulfide releases LC into the cytosol (Montal, 2010; Montecucco & Schiavo, 1994). Once in the cytosol, LC cleaves SNARE proteins with high site specificity, preventing assembly of the fusion complex and blocking neurotransmitter release (Montecucco & Schiavo, 1994; Blasi et al., 1993a; Schiavo et al., 1992).

The following table summarises canonical SNARE targets for serotypes A–G

BoNT serotype	Principal SNARE target(s)	Representative primary evidence
A	SNAP-25	Blasi et al. (1993a); Schiavo et al. (1993)
B	VAMP/synaptobrevin	Schiavo et al. (1992)
C	Syntaxin; also SNAP-25	Blasi et al. (1993b); (additional cellular evidence in later work)
D	VAMP/synaptobrevin	Yamasaki et al. (1994)
E	SNAP-25	Schiavo et al. (1993); Binz et al. (1994)
F	VAMP/synaptobrevin	Yamasaki et al. (1994)
G	VAMP/synaptobrevin	Schiavo et al. (1994)

Mechanistically, cleavage of these SNARE components leads to failure of synaptic vesicle fusion and neurotransmitter exocytosis, producing flaccid paralysis due to blockade of acetylcholine release at neuromuscular junctions and other peripheral cholinergic synapses (Montecucco & Schiavo, 1994; European Centre for Disease Prevention and Control, 2022). BoNT cleavage sites on SNARE complex components are highly specific in location and prevent the release of acetylcholine at the neuromuscular junction, leading to paralysis (Centurioni et al. 2022).

A.2.4 Toxicology and clinical course

A.2.4.1 Intrinsic toxicological properties and relative potency

BoNT toxicology is primarily explained by the combination of catalytic activity and highly selective neuronal targeting. Once the active light chain (LC) reaches the cytosol of a susceptible nerve terminal, even very small amounts of toxin can cleave large numbers of SNARE molecules, producing a substantial biological effect at low toxin burden (Montal, 2010; Montecucco & Schiavo, 1994). The relevant target tissues are primarily peripheral cholinergic synapses, especially neuromuscular junctions, rather than central brain parenchyma; accordingly, clinical botulism presents as paralysis without primary encephalopathy, consistent with toxin action at peripheral nerve terminals (CDC, 2021; Arnon et al., 2001).

Progenitor toxin complexes (PTCs) modify effective potency in a route-dependent manner rather than altering the catalytic chemistry of the LC. In oral exposure contexts, associated proteins, especially NTNHA and, in larger complexes, haemagglutinin (HA) components, protect BoNT from low pH and proteolysis and can facilitate intestinal uptake, helping explain why complexed toxin may be more hazardous in food-borne settings than naked holotoxin (Gu et al., 2012; Lee et al., 2013). Stability is therefore matrix- and environment-dependent and is influenced by pH, proteolysis, and whether the toxin is present in a PTC form that later dissociates under physiological conditions to release the active 150 kDa neurotoxin (Gu et al., 2012; Eisele et al., 2011).

Approximate lethal-dose estimates have been cited in the range of about **1 µg/kg orally, 10 ng/kg by inhalation, and 1 ng/kg intravenously or intramuscularly**, illustrating the strong route dependence of toxicity (Arnon et al., 2001; CDC, 2021).

A.2.4.2 Clinical syndromes and presentation

Major natural botulism syndromes reflect distinct pathobiologies: **food-borne botulism** results from intoxication with preformed toxin, **infant botulism** reflects intestinal colonisation with in situ toxin production, and **wound botulism** results from toxin production in anaerobic wound tissue followed by systemic absorption (CDC, 2021; Arnon et al., 1977; Werner et al., 2000).

Despite these different exposure contexts, the shared clinical phenotype is an acute afebrile neuroparalytic syndrome that typically begins with cranial nerve and bulbar dysfunction and then progresses in a symmetric, descending pattern. Early manifestations commonly include blurred or double vision, ptosis, ophthalmoparesis, dysarthria, dysphagia, dysphonia, dry mouth, and facial weakness, followed by neck weakness, limb weakness, and progressive respiratory compromise (Arnon et al., 2001; Hughes et al., 1981). In food-borne botulism, gastrointestinal symptoms such as nausea, vomiting, abdominal cramps, and sometimes diarrhea may precede the neurologic syndrome, whereas wound botulism usually lacks a gastrointestinal prodrome and may initially be overlooked, particularly when local wound findings are minimal or masked by other clinical issues (Hughes et al., 1981; Werner et al., 2000). Infant botulism often follows a more insidious course, with constipation, poor feeding, weak cry, pooled oral secretions, cranial nerve deficits, generalized hypotonia, and occasional apnea, reflecting intestinal colonisation and in vivo toxin production rather than ingestion of preformed toxin (Arnon et al., 1977).

A.2.4.3 Temporal course, treatment, and outcome

Botulism does not usually cause immediate collapse. Rather, clinically significant neurologic illness typically evolves over hours to days, and for food-borne or aerosol exposure, severe symptoms most

often become evident **within about 12–72 hours**, although the interval varies with dose and route of exposure (Arnon et al., 2001; Cai et al., 2007). As paralysis descends, bulbar dysfunction, upper-airway compromise, and diaphragmatic weakness can culminate in respiratory failure and death unless antitoxin and intensive supportive care are provided in time (Arnon et al., 2001).

Treatment is time-critical and toxin-centric. Equine antitoxin can neutralise circulating toxin but does not reverse established neuronal blockade, while supportive care, often including mechanical ventilation, can be life-saving during prolonged recovery (CDC, 2021; Arnon et al., 2001). Recovery is typically slow rather than abrupt, because neuromuscular transmission must be re-established at intoxicated nerve terminals through synaptic recovery and reinnervation; in severe cases this process may require weeks to months of supportive care, and persistent morbidity has been documented after major outbreaks (CDC, 2021; Mann et al., 1981). This staged progression is relevant because the interval between exposure, first bulbar symptoms, hospital presentation, and respiratory decline can help distinguish point-source intoxication from colonisation-based disease and narrow the likely exposure window (Arnon et al., 2001; Werner et al., 2000).

A.2.5 Exposure, scenarios and hazard assessment

A.2.5.1 Routes of exposure and route-specific toxicological implications

Recognised exposure routes include ingestion of preformed toxin (food-borne botulism), wound contamination with subsequent in situ toxin production, intestinal colonisation in infants and, more rarely, predisposed adults, iatrogenic exposure during medical use, and, rarely, inhalation (CDC, 2021; Arnon et al., 2001; WHO, n.d.). Importantly, BoNT does not penetrate intact skin, so dermal contact without ingestion or mucosal exposure is not a typical intoxication pathway (CDC, 2021; Arnon et al., 2001).

Route strongly modulates realised risk because it determines both whether toxin reaches susceptible nerve terminals and whether physiological barriers, such as gastric acidity and proteolysis, degrade toxin before absorption (CDC, 2021; Gu et al., 2012). In oral exposure, toxicity is closely tied to food matrices and PTC protection, because NTNHA and HA components can protect BoNT during gastric transit and support intestinal uptake (Gu et al., 2012; Lee et al., 2013). In wound botulism, toxin is produced locally in anaerobic tissue and then absorbed systemically, thereby bypassing gastrointestinal degradation (Werner et al., 2000; CDC, 2021). Inhalational botulism is rare in natural settings and is primarily described in occupational, accidental, or deliberate-release contexts; once absorbed, however, the clinical syndrome resembles other forms of botulism (Arnon et al., 2001; CDC, 2021). Iatrogenic exposure arises from therapeutic preparations and is generally linked to dosing, administration, or product misuse rather than natural toxigenesis (Schantz & Johnson, 1992; CDC, 2021).

A.2.5.2 Realistic exposure scenarios

Plausible public-health scenarios are dominated by naturally occurring pathways: inadvertent toxin formation in inadequately processed or stored foods; environmental spore exposure followed by infant intestinal colonisation; and wound contamination in traumatic injuries or in association with injecting-drug use (CDC, 2021; WHO, n.d.; Werner et al., 2000). Less commonly, exposure may arise iatrogenically from medical use of BoNT preparations.

Deliberate release remains a security concern because BoNT combines very high potency with the potential to overwhelm intensive-care resources, but discussion of such scenarios is best oriented

toward consequence management, including clinical recognition, antitoxin access, and ventilator demand, rather than dissemination mechanics (Arnon et al., 2001; CDC, 2021).

A.2.5.3 Hazard assessment

BoNT hazard is shaped by four interacting features: (i) the extreme potency of active toxin, (ii) the potential for prolonged ventilatory and ICU requirements in severe cases, (iii) strong route dependence due to physiological barriers and PTC-mediated protection, and (iv) substantial molecular diversity across serotypes, subtypes, and mosaic forms (Arnon et al., 2001; CDC, 2021; Gu et al., 2012; Dorner et al., 2013; Peck et al., 2017). Taken together, these features make BoNT a high-consequence toxin not only because of lethality risk, but also because severe cases may generate disproportionate healthcare burden relative to case numbers (Arnon et al., 2001; CDC, 2021).

A.2.6 Regulatory significance

BoNT (often phrased as "botulinum neurotoxin/botulinum toxin") is regulated as a high-consequence toxin in multiple control regimes.

- At the treaty level, the **Biological Weapons Convention (BWC)** establishes a prohibition framework for biological agents and toxins "of types and in quantities that have no justification for prophylactic, protective or other peaceful purposes," which is directly relevant to BoNT as a toxin family (United Nations Office for Disarmament Affairs, n.d.).
- Botulinum toxins appear on export-control lists such as the European Union dual-use framework (European Union, 2021) and the Australia Group list of human and animal pathogens and toxins (Australia Group, 2024).
- In the United States, botulinum neurotoxin is included under the Federal Select Agent Program's toxin oversight framework and is treated as a high-priority (Tier 1) toxin in associated guidance (Federal Select Agent Program, 2022).

A.2.7 Summary relevance to EMBRACE

BoNTs are analytically demanding targets for an EMBRACE-style toxin panel because of (i) high potency requiring sensitive methods, (ii) extensive serotype/subtype/mosaic diversity, (iii) frequent occurrence in complex matrices (food, clinical, environmental), and (iv) the need to distinguish *active toxin* from inactive antigen or gene presence (Dorner et al., 2013; Kalb et al., 2015; Peck et al., 2017).

A robust and defensible analytical "stack" typically combines:

- **Broad screening** by immunoassay (antigen) where rapid triage is needed, designed to minimise subtype blind spots (Dorner et al., 2013).
- **Activity confirmation** using Endopep-MS (or equivalent cleavage-product monitoring) as the primary confirmatory evidence for catalytically active BoNT (Boyer et al., 2005; Kalb et al., 2015).
- **Serotype/subtype discrimination** by targeted LC–MS/MS peptide mapping with immunoaffinity enrichment, enabling higher-confidence identification and potentially informative subtype discrimination where databases support it (Kalb et al., 2012; Morineaux et al., 2015).
- **Optional organism-centred testing** (PCR/WGS) when spores/organisms are plausible and when epidemiology or attribution requires strain context—explicitly recognising this does not substitute for toxin confirmation (Lindström & Korkeala, 2006; Hill et al., 2007).

A.3 Saxitoxin and paralytic shellfish toxins

A.3.1 Identity and general description

Saxitoxin (STX) is the parent compound of the paralytic shellfish toxins (PSTs), a family of structurally related guanidinium alkaloids that comprise more than fifty naturally occurring analogues. These include saxitoxin itself together with neosaxitoxin, gonyautoxins, decarbamoyl derivatives, N-sulfocarbamoyl derivatives, and several less common analogues described from marine and freshwater systems (Wiese et al., 2010). Within this structurally diverse family, congeners differ in substituents at key positions on the tetrahydropurine core, which affects charge distribution, physicochemical behaviour, and toxicity (Schantz et al., 1975; Rogers & Rapoport, 1980; Shimizu et al., 1981).

PSTs are notable because they occur across both marine and freshwater environments. In marine systems they are produced primarily by dinoflagellates of the genera *Alexandrium*, *Gymnodinium*, and *Pyrodinium*; in freshwaters, structurally related toxins are produced by several cyanobacterial genera including *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis* / *Raphidiopsis*, *Planktothrix*, and *Lyngbya* (Cusick & Sayler, 2013; Wiese et al., 2010). This dual occurrence explains why the toxin is relevant both to marine food safety and to freshwater/drinking-water risk management. In the framework of the Chemical Weapons Convention, however, only saxitoxin itself is specifically listed in Schedule 1, even though real-world samples commonly contain complex mixtures of PST congeners rather than STX alone (Harju et al., 2015; Dorner et al., 2016).

A.3.2 Biological origin and environmental occurrence

Marine PSTs are formed during blooms of toxigenic dinoflagellates and then transferred through aquatic food webs, especially via filter-feeding shellfish and other marine organisms. Freshwater PSTs arise from cyanobacterial blooms and may contaminate lakes, reservoirs, and potentially raw drinking water sources (Cusick & Sayler, 2013; Gawankar et al., 2024). The movement of PSTs through aquatic food chains is well documented, and numerous traditional as well as non-traditional vectors have been described, including bivalves, gastropods, crustaceans, planktivorous fish, and pufferfish (Deeds et al., 2008; Landsberg et al., 2006).

Occurrence is therefore best understood not as isolated toxin production, but as an ecological and food-chain phenomenon. Human illness is usually associated with contaminated seafood, especially shellfish, but freshwater exposure scenarios are increasingly relevant in the context of cyanobacterial blooms and drinking-water protection (Etheridge, 2010; Gawankar et al., 2024). Monitoring programmes have substantially reduced fatalities in regulated food systems, but PSTs remain globally important as foodborne and environmental toxins (Alexander et al., 2009; Cusick & Sayler, 2013).

A.3.3 Mechanism of action

The principal toxic action of saxitoxin and related PSTs is blockade of **voltage-gated sodium channels** in excitable tissues. STX binds at receptor site 1 on the sodium channel α-subunit and prevents sodium influx through the pore, thereby blocking action potential initiation and propagation in nerves and muscles (Hille, 1975; Catterall et al., 1979; Catterall, 1980; Strichartz, 1984). Functionally, this leads to

failure of neuromuscular transmission and, in sufficiently severe intoxication, flaccid paralysis and respiratory arrest.

Although sodium channels are the best-established target, saxitoxin has also been shown to interact with other ion channel systems under experimental conditions. Effects on potassium and calcium channels have been reported, but the dominant toxicological significance in human poisoning remains sodium-channel blockade (Wang et al., 2003; Su et al., 2004; Cusick & Sayler, 2013). The toxic action is therefore rapid, highly specific at the molecular level, and potentially fatal when respiratory musculature is compromised.

A.3.4 Toxicological properties and relative potency

Saxitoxin is among the most potent naturally occurring low-molecular-weight neurotoxins. Classical mouse toxicology reported an **intraperitoneal LD_{50} of about 10 µg/kg body weight** for STX (Wiberg & Stephenson, 1960). Later work confirmed that potency depends strongly on both **route of exposure** and toxin congener, and that different PST analogues can vary substantially in acute toxicity (Genenah & Shimizu, 1981; Alexander et al., 2009; Munday et al., 2013).

The PST family contains analogues of differing toxic potency, and regulatory toxicology therefore often expresses total toxicity as **saxitoxin equivalents (STXeq)** using toxicity equivalency factors. In the EFSA framework, examples include TEFs of 1.0 for STX, neoSTX, GTX1, and dcSTX; 0.7 for GTX4; 0.6 for GTX3; 0.4 for GTX2 and dcneoSTX; 0.2 for dcGTX2; and 0.1 for GTX5, GTX6, C2, and C4 (Alexander et al., 2009). This is important because environmental and food samples usually contain toxin mixtures rather than a single analyte, and total hazard therefore depends on the congener profile as well as on total toxin concentration.

A.3.5 Human toxicity and clinical course

Human toxicity data are derived mainly from **paralytic shellfish poisoning (PSP)** outbreaks. These data show a clear relationship between ingested dose and clinical severity. Integrated analyses of outbreak material indicate approximate oral doses associated with a 10% probability of specific outcome categories of about **1.85 µg STXeq/kg body weight** for low-severity effects and **5.16 µg STXeq/kg body weight** for moderate-severity effects, with high-severity outcomes occurring at higher doses (Alexander et al., 2009). Considerable interindividual variation exists, reflecting differences in toxin composition, absorbed dose, body weight, age, and access to rapid supportive treatment. Clinical reports also suggest that children may be more susceptible than adults on a body-weight basis (Gessner et al., 1997; Coleman et al., 2018).

Low-severity poisoning typically begins within minutes to a few hours after ingestion and is characterised by tingling or numbness around the lips and mouth, often spreading to the face and neck. Nausea, vomiting, diarrhoea, headache, dizziness, and distal paraesthesiae may also occur. Moderate intoxication is associated with more extensive neurological dysfunction, including limb weakness, incoordination, dysarthria, and ataxia. In severe poisoning, generalized neuromuscular failure develops, progressing to flaccid paralysis and respiratory insufficiency; if untreated, death may occur from respiratory arrest (Etheridge, 2010; Suarez-Isla, 2015).

A commonly cited estimate for a potentially lethal total adult dose is on the order of **1–4 mg STX**, depending on body size, toxin mixture, and medical intervention (Suarez-Isla, 2015). There is **no**

specific antidote for human PSP. Management is supportive, with particular emphasis on airway protection and assisted ventilation during the acute phase. When adequate respiratory support is available, patients who survive the initial phase usually recover rapidly and completely (Etheridge, 2010; Suarez-Isla, 2015; Coleman et al., 2018).

A.3.6 Toxicity by route of exposure

Animal studies demonstrate that the apparent potency of saxitoxin changes markedly with exposure route. In mice, acute oral effect levels are far higher than parenteral effect levels. Oral median toxic doses have been reported at roughly **260–263 µg/kg body weight**, and an oral no-observed-adverse-effect level around **163 µg/kg body weight** has been described in some studies, whereas intraperitoneal and intravenous lethality occurs in the **single-digit to low double-digit µg/kg** range (Wiberg & Stephenson, 1960; Munday et al., 2013). Thus, systemic exposure is markedly more potent than oral exposure.

Experimental inhalation studies in mice indicate still greater apparent potency through the respiratory route. In short nose-only aerosol exposures, a **24-hour LC_{50} of about 0.3 µg STX/L air** was reported, with rapid onset of severe neuromuscular signs. On a retained-dose basis, inhalation exposure was estimated to be substantially more potent than oral exposure and more potent than systemic injection, consistent with rapid bioavailability and direct impairment of respiratory neuromuscular function rather than primary lung injury. From an integrated hazard perspective, inhalation can therefore be considered a particularly efficient route of intoxication.

A.3.7 Public health relevance

PSTs are responsible for **paralytic shellfish poisoning**, one of the best known and most serious marine toxin syndromes. Outbreaks have been described globally and historically have caused both severe illness and death. Monitoring systems for shellfish harvesting areas have substantially reduced incidence in regulated settings, but the hazard remains significant because blooms are episodic, toxin profiles vary, and intoxication may develop rapidly (Etheridge, 2010; Cusick & Sayler, 2013).

The public-health importance of PSTs extends beyond shellfish. Saxitoxin pufferfish poisoning has been documented in the United States in connection with *Pyrodinium bahamense* and contaminated pufferfish, illustrating that intoxication can arise through vectors other than bivalves (Landsberg et al., 2006). Freshwater occurrence is increasingly relevant in bloom-affected lakes and reservoirs, and drinking-water authorities have recognised saxitoxins as an emerging concern, particularly in regions with recurrent cyanobacterial blooms (Gawankar et al., 2024).

A.3.8 Routes of exposure

The dominant natural route of human exposure is **oral ingestion** of contaminated seafood, especially shellfish, and in some instances contaminated fish or pufferfish (Etheridge, 2010; Landsberg et al., 2006). Freshwater exposure may occur through contaminated untreated water or inadequately treated drinking water where cyanobacterial blooms are present (Gawankar et al., 2024). Recreational exposure is also possible in bloom-impacted waters, although oral consumption remains the main route associated with clinically significant intoxication.

From a broader hazard perspective, **inhalational exposure** is toxicologically plausible and experimentally highly potent, although it is not a typical route in natural foodborne intoxication. Parenteral exposure is also highly potent experimentally but is not relevant to normal environmental exposure scenarios.

A.3.9 Methods of dissemination and realistic exposure scenarios

In natural settings, dissemination occurs via **toxin-producing algal or cyanobacterial blooms**, subsequent contamination of aquatic organisms, and transfer through the food web. Shellfish accumulation remains the classic pathway to human exposure, while freshwater dissemination involves cyanobacterial proliferation in lakes and reservoirs and possible transfer into raw water abstractions (Cusick & Sayler, 2013; Gawankar et al., 2024).

From a hazard-assessment perspective, saxitoxin has long been recognised as a potential threat agent because of its high potency and regulatory significance. At the same time, large-scale deliberate dissemination is generally considered unlikely in practice because production of sufficient quantities of purified material, stabilisation, and effective delivery would be technically demanding (Szinicz, 2005; Anderson, 2012; Dorner et al., 2016).

For EMBRACE purposes, PSTs are particularly relevant because they represent a class of **small, non-proteinaceous, highly polar toxins** that can occur in environmental, food, and water-related scenarios. Saxitoxin itself serves as the canonical representative of this family, but the broader PST group must be considered when assessing toxicological significance, exposure potential, and incident interpretation.

A.3.10 Regulatory significance

Within international control frameworks, saxitoxin is specifically listed in Schedule 1 of the Chemical Weapons Convention, which makes it unusual among naturally occurring marine toxins. In food safety, total PST content is regulated in shellfish using STX-equivalent approaches, with the widely established regulatory limit of **800 µg STXeq/kg shellfish meat** derived historically from the mouse bioassay framework (Alexander et al., 2009).

For drinking water, much lower values apply. The **WHO acute guideline value** for total saxitoxins in drinking water is **3 µg/L**, while lower state or national trigger values have also been used, for example **1 µg/L** in Oregon and **1.6 µg/L** in Ohio; New Zealand has also applied **3 µg/L** as a drinking-water maximum acceptable value (Farrer et al., 2015; Ohio EPA, 2020; World Health Organization, 2022). These low values reflect the high potency of STX and the need for sensitive detection and risk management in affected water systems.

A.3.11 Summary relevance to EMBRACE

Saxitoxin and the PST family are relevant to EMBRACE because they represent a major class of **low-molecular-weight neurotoxins** with well-established public-health significance, documented environmental occurrence, diverse natural congeners, and high acute toxicity. They are important not only as marine foodborne toxins, but also as freshwater and drinking-water hazards. Their inclusion in EMBRACE therefore ensures coverage of a toxin family that is toxicologically serious, environmentally relevant, and operationally important in both food and water contexts

A.4 Trichothecenes

A.4.1 Identity, structural features, and toxicological significance

Trichothecenes are a large family of sesquiterpenoid mycotoxins defined by a common 12,13-epoxytrichothec-9-ene core. That epoxide is central to their biological activity and is one of the key structural features underlying their toxic mechanism. Early mechanistic work showed that 12,13-epoxytrichothecenes inhibit eukaryotic protein synthesis at the ribosomal peptidyl-transferase level, establishing the ribosome as a primary toxicological target (Schindler et al., 1974).

From a toxicological perspective, the most relevant food- and feed-associated trichothecenes are usually divided into type A and type B compounds. Type A trichothecenes include T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS), neosolaniol, T-2 triol, and T-2 tetraol, whereas type B trichothecenes include deoxynivalenol (DON), nivalenol (NIV), fusarenon-X, and acetylated DON derivatives. The distinction is not merely classificatory: type A compounds, especially T-2 toxin, are generally regarded as among the most acutely potent trichothecenes in mammals and birds, with particularly prominent dermal, gastrointestinal, hematopoietic, and immunotoxic effects (Shifrin & Anderson, 1999).

Structural substitutions strongly influence toxicological behaviour. For example, the acetylation pattern and ester side chains of T-2 toxin increase lipophilicity relative to more polar metabolites such as HT-2 toxin, T-2 triol, or T-2 tetraol, which affects absorption, tissue distribution, and metabolism. At the same time, metabolic deacetylation does not necessarily abolish toxicity: HT-2 remains toxicologically important and is commonly considered together with T-2 in toxicological assessment (Zhang et al., 2018).

A further toxicological complication is that trichothecenes occur not only as parent toxins but also as modified forms generated by fungal, plant, microbial, or animal metabolism. These include hydrolysed, deepoxy, glucosylated, sulfated, and thiol-conjugated derivatives. Some modified forms are less toxic than the parent compound, whereas others can act as exposure biomarkers or may reconvert to toxic parent structures in vivo or during digestion. This is why toxicological interpretation increasingly treats trichothecenes as families of related toxicants, not isolated single compounds (McCormick et al., 2015; Stanic et al., 2015; Stanic et al., 2016a; Uhlig et al., 2016).

A.4.2 Mechanisms of toxicity

The core toxicological mechanism of trichothecenes is inhibition of eukaryotic protein synthesis. Early biochemical studies demonstrated that T-2 toxin and related trichothecenes inhibit translation and interfere with peptidyl-transferase function on the ribosome (Schindler et al., 1974).

Later mechanistic work showed that trichothecene toxicity is not limited to bulk translational arrest. Shifrin and Anderson (1999) demonstrated that selected trichothecenes trigger a ribotoxic stress response, activating JNK and p38 MAP kinase pathways and promoting apoptosis. This mechanistic framework helps explain why rapidly proliferating and high-turnover tissues such as intestinal epithelium, hematopoietic tissue, lymphoid tissue, and skin are particularly vulnerable.

For T-2 toxin specifically, downstream effects include apoptosis, oxidative stress, inflammatory signalling, impaired barrier tissues, and immunosuppression. In vivo and cell-based studies have linked T-2 exposure to lesions in gastrointestinal, lymphoid, hematopoietic, dermal, and cardiac tissues, consistent with the combination of protein synthesis inhibition and stress-signalling-mediated injury (Jaćević et al., 2020; Shifrin & Anderson, 1999).

T-2 and HT-2 also produce pronounced feeding-related toxicity, including anorexia and emesis. Experimental studies indicate that these responses are associated with gut-brain peptide signalling, including PYY3-36, GIP, and GLP-1, providing a mechanistic basis for feed refusal and emetic symptoms observed in exposed animals (Zhang et al., 2017, 2022).

A.4.3 Target organs and major toxic effects

The gastrointestinal tract is one of the most consistent target systems for trichothecenes. T-2 toxin causes irritation, ulceration, necrosis, and haemorrhagic lesions in the oral cavity and upper gastrointestinal tract. In broiler chickens, Wyatt et al. (1973) showed that severe oral lesions were a primary effect of dietary T-2 exposure and likely contributed directly to reduced feed intake and secondary systemic decline.

Comparable lesion-driven toxicity has been reported in other species. In growing pigs, dietary T-2 reduced feed intake, growth, and altered clinical and metabolic status, while parallel work documented leukocyte effects consistent with immunotoxicity (Rafai et al., 1995a, 1995b). In naturally exposed sheep, intermittent consumption of T-2-contaminated feed produced anorexia, ruminal atony, soft faeces, gastrointestinal inflammation, and substantial mortality (Ferreras et al., 2013).

Hematopoietic and lymphoid tissues are also highly sensitive. This aligns well with the mechanistic expectation that rapidly dividing cell populations are especially vulnerable to ribotoxic and apoptotic injury. Experimental and field studies describe leukopenia, lymphoid depletion, immune suppression, and increased susceptibility to infection following T-2 exposure (Rafai et al., 1995b).

In pigs, Rafai et al. (1995b) documented immune-system effects in growing animals exposed to dietary T-2 toxin, including reduced leukocyte parameters. Historical descriptions of severe human trichothecene poisoning, especially alimentary toxic aleukia, also centred on hematological collapse and mucosal haemorrhage, although those episodes are historically complex and not documented with modern analytical certainty.

Among trichothecenes, T-2 toxin is particularly notable for **dermal toxicity**. It can injure intact skin and produce erythema, inflammation, blistering, and necrosis. This is toxicologically important because it distinguishes T-2 from many foodborne toxins that act mainly after ingestion. Experimental work and later mechanistic studies indicate that dermal injury reflects a combination of local cytotoxicity, oxidative stress, MAPK activation, and apoptosis (Shifrin & Anderson, 1999).

This strong cutaneous activity is one reason T-2 has long attracted attention in military, forensic, and deliberate-release discussions, even though evidence for actual weaponised field use has remained controversial.

Cardiac and other systemic toxicity. Although gastrointestinal, immune, and dermal effects dominate most descriptions, T-2 toxin can also affect other organ systems. In rats, a single

subcutaneous dose produced cardiomyopathic changes with histopathological evidence of myocardial injury, indicating that the toxin is not solely a mucosal or hematopoietic toxicant (Jaćević et al., 2020).

Reproductive, developmental, and fetal toxicity have also been described in experimental systems and are consistent with the broader concept that T-2 targets actively proliferating or metabolically sensitive tissues.

A.4.4 Species differences and comparative sensitivity

Species sensitivity differs markedly. Poultry are notably sensitive to oral lesions and feed refusal caused by T-2 toxin, with clinically relevant effects reported at low mg/kg feed concentrations (Wyatt et al., 1973). Pigs are also sensitive, with decreased intake, reduced growth, hematological changes, and gastrointestinal effects observed in feeding studies (Rafai et al., 1995a, 1995b).

Ruminants are often considered somewhat less sensitive to some mycotoxins because of microbial metabolism in the rumen, but T-2 still causes important disease under real exposure conditions, as shown by naturally occurring sheep intoxication (Ferreras et al., 2013). In rodents, mechanistic and dose-response studies have been especially useful for defining apoptosis, ribotoxic stress, anorexia, emesis-related signalling, and systemic target-organ effects (Jaćević et al., 2020; Shifrin & Anderson, 1999; Zhang et al., 2017, 2022).

A.4.5 Human relevance

The best-known historical human syndrome associated with trichothecenes is alimentary toxic aleukia (ATA), a severe disease linked to overwintered mould-contaminated grain consumed in the former USSR. The syndrome included gastrointestinal irritation, leukopenia, mucosal bleeding, secondary infection, and high mortality. Modern attribution specifically to T-2 toxin and related trichothecenes is based on later toxicological and mycological interpretation rather than the type of analytically definitive evidence expected in contemporary incident investigation.

T-2 exposure has also been discussed in relation to Kashin–Beck disease, but the causal status is far less certain. The association is biologically plausible and has been investigated epidemiologically and metabolically, yet it remains much weaker than the historical association with ATA.

Overall, the human relevance of trichothecenes is clear in food and feed safety and in potential deliberate contamination scenarios, but well-documented acute human poisonings are rare in modern literature compared with the animal evidence base.

A.4.6 Quantitative toxicology and dose-response

For toxicological assessment, T-2 and HT-2 are commonly considered together because T-2 is rapidly metabolised to HT-2 and both contribute materially to toxicity (Zhang et al., 2018).

In poultry, dietary concentrations around **0.5–1 mg/kg feed** can already induce oral mucosal lesions, while **2–6 mg/kg feed** have been associated with reduced feed intake, impaired growth, and more overt toxicosis (Wyatt et al., 1973). In pigs, feeding studies have reported reduced feed intake and performance at **0.5 mg/kg feed** and more severe clinical effects at **5–10 mg/kg feed** (Rafai et al., 1995a).

In sheep, a field outbreak associated with naturally contaminated feed caused both acute and chronic disease, including substantial mortality, demonstrating that real-world intermittent exposure can be severe even when exact individual dose reconstruction is difficult (Ferreras et al., 2013). In rats, **0.23 mg/kg subcutaneous** has been used experimentally as approximately an **LD50-equivalent** dose in cardiotoxicity studies, illustrating the potency of acute systemic exposure in that model (Jaćević et al., 2020).

No reliable human LD50 has been established for T-2 or HT-2 toxin. Available quantitative acute-toxicity data are derived from animal studies and indicate marked route-, species-, and age-dependence. Oral LD50 values for T-2 toxin in experimental animals are in the low mg/kg range, while parenteral exposure may be more potent. T-2 toxin is notable in that systemic toxicity may occur after oral, dermal, or inhalational exposure."

The main quantitative message is that clinically important trichothecene toxicity occurs across a broad spectrum: from subacute feed-level exposures causing intake suppression, oral lesions, and immune effects, to more acute systemic exposures causing severe organ injury and, historically, fatal poisoning syndromes.

A.4.7 Toxicological implications for EMBRACE

For EMBRACE, the toxicology of trichothecenes has several practical implications.

First, the class should not be framed only as a food-safety issue. T-2 toxin in particular has mucosal, dermal, hematological, and systemic toxicity consistent with relevance in deliberate contamination and forensic scenarios (Shifrin & Anderson, 1999).

Second, because T-2 rapidly forms HT-2 and additional metabolites, toxicological interpretation should account for parent-plus-metabolite exposure, not parent toxin alone (Zhang et al., 2018).

Third, symptom patterns such as oral lesions, feed refusal, vomiting or emesis, leukopenia, mucosal haemorrhage, and skin necrosis are toxicologically coherent with the mechanistic and animal evidence base and may help inform matrix choice and biomarker strategy in incident investigation (Ferreras et al., 2013; Rafai et al., 1995a, 1995b; Wyatt et al., 1973).

Fourth, the existence of modified forms and biomarkers supports the value of a broadened analytical scope in biological and environmental samples, especially when exposure reconstruction or attribution is important (McCormick et al., 2015; Stanic et al., 2015; Stanic et al., 2016a, 2016b; Uhlig et al., 2016).

A.5 Microcystins and nodularin

A.5.1 Overview and toxicological relevance

Microcystins (MCs) and nodularins (NODs) are closely related **cyanobacterial hepatotoxins** of major environmental and public-health relevance. Microcystins are monocyclic heptapeptides, whereas nodularins are structurally related cyclic pentapeptides (Pearson et al., 2010; Schreidah et al., 2020). Despite this difference in ring size, both toxin groups share key structural features, most notably the hydrophobic β-amino acid **Adda** (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, which is central to biological activity and therefore also analytically important (Rinehart et al., 1988; Pearson et al., 2010). Their toxicological significance arises from a combination of potent acute hepatotoxicity, broad environmental occurrence, multiple exposure routes, and evidence that repeated or chronic exposure may contribute to longer-term pathological effects, including tumor promotion (Yoshizawa et al., 1990; Ohta et al., 1994; Fujiki & Suganuma, 2011).

Microcystins are produced by several bloom-forming cyanobacterial genera, especially in freshwater systems, whereas nodularin is classically associated with *Nodularia spumigena* in brackish waters such as the Baltic Sea, although freshwater occurrences have also been reported (Pearson et al., 2010; Chen et al., 2013). Because both toxin classes act through closely related mechanisms and share broad toxicological features, they are commonly discussed together. At the same time, there are important differences in exposure evidence: microcystins are supported by a much larger experimental and epidemiological literature, whereas nodularin toxicology is still more heavily inferred from animal work and mechanistic similarity (Chen et al., 2013; Melaram et al., 2024).

A.5.2 Structural features relevant to toxicity

The general microcystin scaffold is usually described as cyclo-(D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha), where X and Z are variable L-amino acids; the best-known congener, **MC-LR**, contains leucine and arginine at these positions (Pearson et al., 2010; Schreidah et al., 2020). Nodularin has an analogous but smaller cyclic structure containing D-MeAsp, L-Arg, Adda, D-Glu, and N-methyldehydrobutyrine (Mdhb/MeDhb) (Pearson et al., 2010; Chen et al., 2013). The structural similarity explains why both toxin groups bind to the same molecular targets and often behave similarly in functional assays.

Several structure–activity relationships are established. The Adda side chain is indispensable for potent protein phosphatase inhibition; modifications in Adda can markedly reduce toxicity (Rinehart et al., 1988; Pearson et al., 2010). In microcystins, the Mdha residue can participate in covalent interaction with protein phosphatases, helping stabilize inhibition, although this covalent step is not the sole determinant of potency (MacKintosh et al., 1995; Craig et al., 1996; Schreidah et al., 2020). Congener-specific toxicity differs considerably: MC-LR is among the most studied and often among the more potent common congeners, whereas MC-RR is less acutely toxic in mice, and more hydrophobic congeners such as MC-LF and MC-LW may show high cellular toxicity in some models due to altered membrane transport and intracellular behavior (Pearson et al., 2010; Díez-Quijada et al., 2019).

A.5.3 Toxicokinetics and organotropism

A defining feature of MC and NOD toxicology is that these compounds are not freely membrane-permeable in a nonspecific manner, but instead rely importantly on active uptake transporters, especially members of the organic anion transporting polypeptide (OATP) family (Runnegar et al., 1991, 1995; Niedermeyer et al., 2014). In mammals, this transporter dependence largely explains their strong hepatic tropism, because liver cells express transport systems that efficiently take up these toxins (Pearson et al., 2010; Arman & Clarke, 2021). OATP1B1 and OATP1B3 are particularly relevant in human liver, although tissue distribution is broader and helps explain documented extrahepatic effects in kidney, intestine, lung, and other organs (Niedermeyer et al., 2014; Melaram et al., 2024).

This transport dependence is toxicologically important in two ways. First, it means that exposure route matters greatly: direct parenteral exposure can be dramatically more dangerous than oral exposure, because absorption barriers are bypassed. Second, it means that congener-specific toxicity does not depend only on intrinsic phosphatase-binding potency, but also on transporter affinity and tissue uptake. Thus, some congeners that are less potent in simple phosphatase inhibition assays may still show pronounced toxicity in cells or tissues with favorable uptake characteristics (Díez-Quijada et al., 2019; Schreidah et al., 2020).

A.5.4 Primary mechanism: inhibition of PP1 and PP2A

The central toxicodynamic mechanism of both microcystins and nodularins is the potent inhibition of **serine/threonine protein phosphatases PP1 and PP2A** (Yoshizawa et al., 1990; Honkanen et al., 1991). These enzymes are critical regulators of intracellular phosphorylation state, cytoskeletal organization, and signal transduction. Toxin binding leads to loss of normal phosphatase activity, followed by widespread hyperphosphorylation of intracellular proteins, disruption of the actin cytoskeleton, altered signaling, and ultimately cell injury or death (Eriksson et al., 1990; Campos & Vasconcelos, 2010).

For microcystins, a two-step interaction model has often been described: an initial high-affinity noncovalent binding event followed by slower formation of a more stable adduct involving the Mdha residue and a cysteine residue in the catalytic subunit (Runnegar et al., 1995; MacKintosh et al., 1995; Craig et al., 1996). Nodularin binds in a closely related manner and strongly inhibits PP1 and especially PP2A, though its interaction is often described as somewhat different in covalent character from canonical microcystins (Pearson et al., 2010; Chen et al., 2013). Regardless of those structural subtleties, the biological consequence is similar: collapse of phosphatase-mediated control.

In hepatocytes, this manifests as cytoskeletal disorganization, rounding and detachment of cells, sinusoidal collapse, intrahepatic hemorrhage, and rapid hepatic failure in severe intoxication (Eriksson et al., 1990; Pearson et al., 2010). This phosphatase inhibition mechanism also underlies the use of **protein phosphatase inhibition assays** as functional or broad-screening methods for both toxin classes, although such assays measure biological activity rather than exact toxin identity (An & Carmichael, 1994).

A.5.5 Acute toxicity and dose–response

The best-known quantitative acute-toxicity figures come from mouse studies. For MC-LR, an often-cited value is an **intraperitoneal LD50 of about 50 µg/kg body weight in mice** (Pearson et al., 2010).

By contrast, MC-RR is markedly less potent in the same type of model, with values on the order of **600 µg/kg** reported in older work summarized by Pearson et al. (2010). For nodularin, a similarly high potency is reported, with an **intraperitoneal LD50 in mice also around 50 µg/kg** (Pearson et al., 2010). These values underscore that both toxin groups must be regarded as highly potent acute hepatotoxins.

However, these figures must be interpreted cautiously. Intraperitoneal LD50 values are useful for comparing congeners and models, but they do not translate directly into human oral toxicity thresholds. Oral exposure generally involves lower bioavailability, matrix effects, and first-pass processes. Accordingly, environmental health guidance is usually expressed through tolerable daily intake and drinking-water guideline values rather than human lethal-dose estimates. For MC-LR, the commonly cited tolerable daily intake is **0.04 µg/kg body weight/day**, and the WHO drinking-water guideline value historically associated with MC-LR is **1 µg/L** (Chorus & Bartram, 1999; Farrer et al., 2015). These are risk-management benchmarks, not symptom thresholds.

In acute poisoning, symptoms are dose-dependent but not tightly quantified in humans. At lower-level environmental exposure, reported symptoms include gastrointestinal upset, nausea, vomiting, diarrhea, mouth or throat irritation, skin symptoms, and respiratory irritation after recreational exposure to aerosols or contaminated water (Backer et al., 2008, 2010; Stewart et al., 2006; Wood, 2016). At higher effective doses, the clinical picture shifts toward **hepatotoxicity**, with elevated liver enzymes, jaundice, hemorrhagic injury, and potentially acute liver failure (Jochimsen et al., 1998; Chen et al., 2009; Li et al., 2011).

A.5.6 Oxidative stress, inflammation, and secondary mechanisms

Although PP1/PP2A inhibition is the primary mechanism, both toxin groups also provoke a broader network of downstream injury responses. A substantial literature supports a role for oxidative stress, including generation of reactive oxygen species, mitochondrial dysfunction, depletion of cellular antioxidant defenses, and activation of stress-signaling pathways such as JNK and MAPK cascades (Campos & Vasconcelos, 2010; McLellan & Manderville, 2017; Schreidah et al., 2020). In experimental systems, these processes contribute to hepatocyte apoptosis, inflammatory signaling, and tissue injury beyond simple phosphatase inhibition.

Microcystins have also been linked to DNA damage and genotoxic responses in several model systems, though the extent to which this reflects direct genotoxicity versus secondary oxidative and signaling injury remains debated (Žegura et al., 2011; Melaram et al., 2024). Extrahepatic effects have been documented in the kidney, lung, intestine, reproductive system, and possibly the nervous system, especially in chronic or repeated-exposure models (Arman & Clarke, 2021; Melaram et al., 2024). Nodularin shows similar downstream effects, including induction of TNF-α, IL-8, MAPK activation, and endoplasmic reticulum stress in hepatocyte-related systems (Sueoka et al., 1997; Meili et al., 2016).

A.5.7 Tumor promotion and chronic effects

Both microcystins and nodularins are widely regarded as tumor promoters rather than classical direct genotoxic carcinogens. In rodent models, microcystin-LR can promote liver tumor formation after prior initiation, and nodularin has shown particularly strong liver tumor-promoting activity in male F344 rats (Nishiwaki-Matsushima et al., 1992; Ohta et al., 1994). Mechanistically, this is plausibly linked

to persistent phosphatase inhibition, dysregulated cell signaling, inflammatory mediators such as TNF-α, and oxidative injury (Fujiki & Suganuma, 2011).

Human evidence is weaker and more epidemiological than mechanistic. Several studies from China and Serbia have reported associations between cyanotoxin-contaminated drinking water and primary liver cancer or related chronic hepatic outcomes, although causal interpretation remains difficult because of co-exposures and study-design limitations (Ueno et al., 1996; Svirčev et al., 2009; Melaram et al., 2024). More recent human studies have linked chronic microcystin exposure to elevated serum liver enzymes and possible kidney effects, especially in combination with other pollutants such as cadmium (Chen et al., 2009; Li et al., 2011; Feng et al., 2022). For nodularin specifically, there is still little direct human epidemiology, and risk characterization remains heavily dependent on animal toxicology and comparison with microcystins (Chen et al., 2013; Melaram et al., 2024).

A.5.8 Human exposure evidence

The clearest severe human poisoning episode involving microcystins is the **Caruaru hemodialysis incident** in Brazil. In that outbreak, patients were exposed to contaminated dialysate, leading to acute liver failure and multiple deaths; microcystins were later identified in liver tissue from fatal cases (Jochimsen et al., 1998). This remains the strongest direct evidence that microcystins can cause catastrophic human poisoning under high systemic exposure.

Outside such extreme events, most documented human exposures are environmental and lower-level. These include drinking-water exposure, recreational contact, possible inhalation of aerosols, and dietary exposure via contaminated aquatic foods or algal supplements (Backer et al., 2008, 2010; Ibelings & Chorus, 2007; Turner et al., 2018). Epidemiological work has reported microcystins in human serum and associations with altered liver enzymes in chronically exposed populations (Chen et al., 2009; Li et al., 2011). Nodularin exposure in humans is less well documented directly, but its occurrence in seafood and brackish-water bloom systems is well established, making it relevant to foodborne and environmental exposure assessment (Van Buynder et al., 2001; Mazur-Marzec et al., 2007; Chen et al., 2013).

A practical toxicological point for monitoring is that **symptoms alone are nonspecific**. Mild gastrointestinal, mucosal, dermatological, or respiratory complaints can occur at relatively low exposure, whereas severe poisoning is dominated by liver injury. Because many cyanobacterial exposure scenarios also involve other irritants or toxins, analytical confirmation is essential when attributing illness to MCs or NODs (Stewart et al., 2006; Wood, 2016).

A.5.9 Implications for analytical and forensic work

From an analytical and forensic perspective, the toxicology of microcystins and nodularins has several implications. First, because toxicity depends on free biologically active toxin, methods that only measure one common congener may underestimate toxic relevance if other congeners are present (Díez-Quijada et al., 2019). Second, because both groups share **PP1/PP2A inhibition**, functional assays can provide useful evidence of total biological activity, but they lack full structural specificity and therefore should be complemented by LC–MS/MS or other confirmatory methods (An & Carmichael, 1994; Schreidah et al., 2020). Third, because exposure can occur in water, food, and

biological samples, toxicological interpretation requires matrix-aware sample preparation and careful distinction between free toxin, conjugated/bound toxin, and broad activity-based readouts.

For EMBRACE-type work, the toxicological literature supports a layered approach: targeted LC–MS/MS for identity-resolved confirmation of key congeners, optionally combined with functional phosphatase-based approaches where broad toxicity screening or total active burden is important. Toxicology also supports prioritizing matrices where real exposure is plausible: raw and treated water, bloom material, seafood and food supplements, and selected biological specimens when exposure assessment is needed.

A.6 Aconitum alkaloids

A.6.1 Identity, structural features, and toxicological relevance

The toxicologically most important Aconitum alkaloids are diterpenoid alkaloids, especially the C19 diterpenoid alkaloids. Within this group, the compounds of greatest human relevance are the diester diterpenoid alkaloids (DDAs), notably aconitine, mesaconitine, and hypaconitine, together with several structurally related congeners such as yunaconitine, crassicauline A, deoxyaconitine, and 10-hydroxymesaconitine (Bisset, 1981; Lai et al., 2006; Chung et al., 2012; Huang et al., 2022). These compounds occur primarily in species of Aconitum, especially roots and processed herbal materials used in traditional medicine, and they are responsible for most severe aconite poisonings reported in clinical and forensic literature (Tai et al., 1992; Lin et al., 2004; Chan, 2014).

Structurally, the most toxic Aconitum alkaloids share a polycyclic norditerpenoid skeleton bearing multiple oxygenated substituents and, in the classical aconitine-like toxins, a characteristic 8-O-acyl / 14-O-aroyl diester arrangement. This ester pattern is strongly associated with high toxicity. Hydrolytic conversion of DDAs to monoester diterpenoid alkaloids (MDAs), such as benzoylaconine, benzoylmesaconine, and benzoylhypaconine, substantially reduces acute toxicity, and further hydrolysis to non-esterified alcohol amine-type alkaloids reduces toxicity still further (Bisset, 1981; Zhang et al., 2017; Huang et al., 2022). This structure–toxicity relationship is central to both traditional processing and toxicological interpretation.

From a toxicological perspective, the important point is that aconite poisoning is not always caused by aconitine alone. Real exposures may involve mixtures of DDAs, MDAs, and non-ester alkaloids whose relative abundance depends on species, plant part, processing history, and formulation. Accordingly, poisoning severity reflects both total alkaloid burden and congener composition, not merely the presence or absence of aconitine itself (Lai et al., 2006; Chung et al., 2012; Wang et al., 2025).

A.6.2 Mechanism of toxicity

Aconitum alkaloids act primarily on **voltage-gated sodium channels** in excitable tissues. Classical studies showed that aconitine binds to activated sodium channels and promotes persistent channel activation, causing prolonged sodium influx and impaired repolarisation (Friese et al., 1997; Gutser et al., 1998). In the myocardium, this produces marked electrophysiological instability, including triggered activity, delayed afterdepolarisations, and malignant ventricular arrhythmias. In

cardiomyocyte models, aconitine has also been shown to disturb intracellular calcium homeostasis, further contributing to arrhythmogenesis and contractile dysfunction (Fu et al., 2007).

This sodium-channel-mediated action explains why the dominant acute toxic syndrome is **combined neurotoxicity and cardiotoxicity**, with the cardiovascular component being the most life-threatening. Autonomic effects may also contribute, including vagally mediated disturbances and hypotension, but severe poisoning is largely driven by direct electrophysiological toxicity in the heart (Tai et al., 1992; Lin et al., 2004; Fu et al., 2006; Zhou et al., 2021).

The toxicological hierarchy within the class is broadly consistent with the structural categories above: **DDAs are the most potent**, MDAs are substantially less toxic, and the alcohol amine-type alkaloids are generally less acutely toxic still, although they may still contribute to overall pharmacological or toxic effects in mixed exposures (Bisset, 1981; Shan et al., 2017; Huang et al., 2022).

A.6.3 Target organs and clinical syndrome

The principal target organs are the heart, peripheral nervous system, and central nervous system. Clinically, poisoning usually begins with gastrointestinal and sensory symptoms, followed by potentially catastrophic cardiovascular complications. Early manifestations commonly include nausea, vomiting, abdominal pain, diarrhoea, paresthesia, numbness or tingling of the lips and extremities, sweating, and weakness. These may progress rapidly to hypotension, conduction abnormalities, ventricular tachycardia, bidirectional ventricular tachycardia, ventricular fibrillation, cardiogenic shock, and death (Tai et al., 1992; Lin et al., 2004; Smith et al., 2005).

The onset after oral exposure is typically rapid, often within **minutes to a few hours**, consistent with efficient gastrointestinal absorption and high intrinsic potency (Tai et al., 1992; Lin et al., 2004). In fatal cases, death is usually attributable to **refractory ventricular arrhythmia** or profound cardiovascular collapse rather than to delayed organ failure (Niitsu et al., 2013).

Post-mortem and animal distribution studies indicate that aconitum alkaloids can be detected in **blood, urine, liver, kidney, heart, and brain**, supporting the observed pattern of systemic toxicity and providing a biochemical basis for both clinical and forensic sampling strategies (Niitsu et al., 2013; Ji et al., 2019; Zhang et al., 2020).

A.6.4 Human relevance and exposure scenarios

Human poisoning is well documented and remains clinically and forensically relevant, especially in settings involving **traditional herbal medicines**, **improper processing**, **overdose**, **misidentification**, or **home-made medicinal liquors and decoctions** (Tai et al., 1992; Lin et al., 2004; Chan, 2014). Poisoning may arise after therapeutic misuse, accidental substitution, or deliberate administration, and both sporadic and outbreak-type events have been reported.

A recurring feature of the clinical literature is that many poisonings reflect failure of detoxifying processing steps intended to hydrolyse DDAs to less toxic forms. This is toxicologically important because it links the hazard directly to chemical speciation: inadequately processed material retains a higher fraction of diester alkaloids and therefore a much greater acute arrhythmogenic potential (Chan, 2014; Zhang et al., 2017; Huang et al., 2022).

For forensic purposes, the literature also shows that different Aconitum species or preparations may yield quite different alkaloid patterns. This complicates exact source attribution, but it does not alter the central toxicological conclusion that high-risk exposure is generally associated with preservation or ingestion of **aconitine-like diester alkaloids** (Lai et al., 2006; Chung et al., 2012; Wang et al., 2025).

A.6.5 Quantitative toxicology

Quantitative toxicology for aconitum alkaloids must be treated cautiously. **A validated human LD50 does not exist**, and values sometimes presented as "human LD50" are in practice extrapolated or inferred from case reports rather than experimentally established data. In humans, the literature is better interpreted in terms of reported toxic or fatal dose ranges and measured biological concentrations than as formal LD50 values.

Clinical literature frequently cites severe poisoning after approximately **0.2 mg oral aconitine** and fatal outcomes after total oral doses in the low milligram range, often around **2–6 mg**, but these figures should be understood as approximate case-based estimates rather than definitive toxicological constants (Tai et al., 1992; Lin et al., 2004; Chan, 2014).

Animal data clearly show strong route dependence and high potency. In mice, reported LD50 values for aconitine are approximately **1.8 mg/kg orally**, **0.27 mg/kg subcutaneously**, **0.31 mg/kg intraperitoneally**, and **0.12 mg/kg intravenously**; corresponding values for mesaconitine and hypaconitine indicate similarly high potency, with mesaconitine often slightly more potent than aconitine in parenteral models and hypaconitine somewhat less potent (Bisset, 1981; Huang et al., 2022). The much lower parenteral LD50 values are consistent with bypass of first-pass metabolism and rapid delivery to the cardiovascular system.

For the less toxic hydrolysis products, the difference is substantial. Reported mouse intraperitoneal LD50 values for benzoylaconine, benzoylmesaconine, and benzoylhypaconine are on the order of tens to hundreds of mg/kg, confirming the major toxicity reduction associated with loss of the diester structure (Bisset, 1981; Huang et al., 2022).

Measured concentrations in human poisoning cases vary widely depending on congener, matrix, and time since exposure. Post-mortem blood or urine may show aconitine, mesaconitine, hypaconitine, yunaconitine, or related alkaloids from low ng/mL to much higher levels, but interpretation is strongly limited by instability, metabolism, variable sampling delay, and the fact that some patients die from rapidly evolving arrhythmia before extensive redistribution occurs (Niitsu et al., 2013; Wang et al., 2025). Accordingly, concentration data are evidentially useful, but there is no simple universal fatal blood threshold.

Overall, the quantitative literature supports four points:

(1) aconite alkaloids are high-potency toxins,

(2) route of exposure strongly influences potency,

(3) diester alkaloids are markedly more toxic than monoester or non-ester forms, and

(4) human interpretation should rely on case-based toxic dose ranges and congener patterns, not on an assumed human LD50.

A.7 Strychnine and related Strychnos alkaloids

A.7.1 Identity, structural features, and toxicological relevance

The toxicological focus in **Strychnos** is centered on **strychnine** and, secondarily, **brucine**. Both are **indole alkaloids** classically associated with *Strychnos nux-vomica* and related materials. Strychnine is the dominant acute toxicant of forensic importance, whereas brucine is structurally related and biologically active but less potent (Duverneuil et al., 2004; Lin et al., 2015).

Structurally, strychnine is a rigid, highly oxygenated polycyclic indole alkaloid with a tertiary amine and a compact cage-like skeleton. Its strong biological activity is linked less to reactive chemistry than to highly specific receptor-level pharmacology in the central nervous system. Brucine differs by additional methoxy substitution and is generally less potent toxicologically, although still relevant in mixed Strychnos exposures (Lin et al., 2015).

In vivo, both parent compounds may undergo metabolic oxidation to N-oxides, including strychnine N-oxide and brucine N-oxide, which are less toxic than the parent alkaloids but toxicologically relevant as metabolites and markers of exposure (Lin et al., 2015). For acute poisoning, however, the parent compound strychnine remains the principal concern.

A.7.2 Mechanism of toxicity

Strychnine acts primarily as a competitive antagonist of inhibitory glycine receptors in the spinal cord and brainstem. By blocking glycinergic inhibition, it removes normal restraint on reflex pathways and produces extreme neuronal hyperexcitability. The result is the classic syndrome of painful generalized muscle spasms, hyperreflexia, opisthotonus, convulsions triggered by minimal stimuli, and respiratory compromise (Perper, 1985; Philippe et al., 2004).

This mechanism differs fundamentally from that of aconitum alkaloids. Strychnine does not primarily cause cardiotoxicity through ion-channel dysregulation; rather, it causes disinhibition of motor pathways and can lead to death by asphyxia, respiratory failure, or secondary metabolic complications associated with repeated severe convulsions (Perper, 1985; Duverneuil et al., 2004).

Brucine appears to share some mechanistic features but is less potent at producing the classical convulsant syndrome. In practical forensic toxicology, the toxicodynamic signature is therefore dominated by strychnine.

A.7.3 Target organs and clinical syndrome

The principal toxicological target is the central nervous system, especially the spinal cord and brainstem inhibitory pathways. The hallmark clinical picture is abrupt onset of marked motor excitability, often with anxiety, restlessness, heightened sensory responsiveness, and rapidly developing muscle spasms. Severe cases show generalized extensor spasms, opisthotonus, clenched jaw, and stimulus-induced convulsions while consciousness may be initially preserved between episodes (Perper, 1985).

This picture is sufficiently characteristic that classical strychnine poisoning has long been recognized clinically and forensically. Fatality commonly results from **respiratory arrest**, **exhaustion**, or the

consequences of sustained convulsive activity rather than from a direct primary cardiac mechanism (Perper, 1985; Duverneuil et al., 2004).

A.7.4 Human relevance and exposure scenarios

Human strychnine poisoning is well documented in accidental, suicidal, and homicidal contexts, as well as through misuse of herbal or traditional preparations containing Strychnos materials (Perper, 1985; Duverneuil et al., 2004). The compound is historically important in forensic toxicology because of both its high potency and its dramatic symptomatology.

In the context of plant-derived toxicants, the main relevance is oral ingestion of **Strychnos-containing material** or preparations derived from it. Because the clinical syndrome is acute and distinctive, diagnosis may be strongly supported by symptoms, but confirmatory toxicology remains important in mixed or delayed cases (Duverneuil et al., 2004).

A.7.5 Quantitative toxicology

As with aconitine, a formal experimental **human LD50 is not available**, and case-based toxic and fatal dose estimates are more appropriate than any purported human LD50 value.

Older toxicological literature commonly places the adult fatal dose of strychnine in the **low tens of milligrams**, often around **30–100 mg**, although susceptibility varies and serious poisoning may occur at lower doses. Blood concentrations below **2 mg/L** have been associated with toxicity, and concentrations in the **2–10 mg/L** range have often been considered potentially lethal in forensic interpretation, but such values must be applied cautiously because of sampling delay, redistribution, and case variability (Perper, 1985; Zhang et al., 2023).

Brucine is less potent than strychnine, and in mixed Strychnos exposures it is usually regarded as a secondary contributor to acute lethality. Pharmacokinetic work in rats further suggests that both strychnine and brucine are absorbed rapidly after oral administration and that co-occurring Strychnos alkaloids may modestly alter their absorption and disposition (Lin et al., 2015). These findings are relevant to interpretation of plant extracts but do not change the central point that strychnine is the dominant acute convulsant hazard.

Overall, the quantitative evidence supports the view that strychnine is a highly potent centrally acting convulsant toxin for which toxicological interpretation should rely on case-based human dose and concentration ranges, not on a supposed human LD50.

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B ANNEX B - ANALYTICAL READINESS LEVEL FRAMEWORK

Purpose of this annex

This annex defines the analytical readiness framework used in this deliverable to describe the maturity of analytical capability for each EMBRACE target biotoxin. Its purpose is to communicate readiness consistently and transparently across the panel without overstating capability. Readiness is expressed in terms of what can currently be delivered as a defensible qualitative analytical outcome.

B.1 Scope and application

Analytical readiness in EMBRACE is matrix-sensitive. In this first iteration, readiness is not assigned exhaustively on a matrix-by-matrix basis. Instead, readiness is reported primarily at target or toxin-class level and interpreted together with the representative matrices examined, the broad operational matrix classes used in the workflow, and the limitation statements in the main report.

A toxin may therefore be confirmation-ready in some analytical contexts without being confirmation-ready across all matrices or all possible sample histories. Screening readiness may also exist before confirmatory readiness. Readiness assignments in this deliverable should therefore be read together with the relevant limitations, QA/QC conditions, and reporting constraints.

The framework applies primarily to detection and identification readiness. Attribution-oriented analysis is considered separately and, in the first iteration, remains preparatory rather than operational.

B.2 Definitions of readiness levels

The following readiness levels are used in this deliverable:

R0 — Not initiated

No practical workflow has yet been established in the current iteration.

R1 — Screening-ready

A screening or triage capability exists, but the evidential chain is not yet sufficient for confirmatory reporting.

R2 — Confirmation under development

A confirmatory workflow has been defined in principle and at least partly implemented, but remains incomplete with respect to matrix coverage, evidential chain, QA/QC structure, or reporting criteria.

R3 — Confirmation-ready

Qualitative identification is achievable with documented orthogonality, QA/QC logic, and reporting basis suitable for defensible interpretation.

These readiness levels apply primarily to detection and identification readiness. Attribution-oriented readiness is assessed separately and, at the present stage, should be understood as preparatory only.

B.3 Evidence requirements per readiness level

To reduce ambiguity, readiness is assigned against documented evidence categories. The minimum evidence expected at each level is outlined below.

R0 — Not initiated

- Target and intended analytical use identified, but no practical workflow established.
- No decision rule or reporting template available for the intended analytical lane.

R1 — Screening-ready

- Screening capability described with a defined purpose.
- Screening result categories defined.
- Minimum QC elements documented for the screening lane.
- Explicit statement that screening outputs are not confirmatory identifications.

R2 — Confirmation under development

- Confirmatory workflow defined in principle and at least partly implemented.
- Intended orthogonal evidential chain identified.
- Draft decision rule and reporting language available.
- QA/QC elements and acceptance logic partly integrated.
- Some within-laboratory performance evidence and/or representative-matrix evidence available, but matrix coverage remains incomplete.

R3 — Confirmation-ready

- Confirmatory workflow documented for the stated target.
- Qualitative decision rule documented and applied consistently.
- Orthogonal evidential basis documented.
- QA/QC elements and run-acceptance logic integrated into interpretation and reporting.
- Within-laboratory evidence sufficient to support consistent qualitative identification.
- Standard limitation language included as part of reporting.

B.4 How readiness is assigned

Readiness is assigned through structured review of the available evidence for each target and analytical workflow. The assignment considers:

- whether the workflow exists and is documented;
- whether qualitative decision rules are defined and stable;
- whether the required orthogonal evidence is available for confirmatory use;
- whether QA/QC elements and acceptance logic are defined and integrated;
- whether the workflow has been demonstrated across representative matrices or matrix classes; and
- whether within-laboratory reproducibility has been demonstrated to a degree appropriate to the maturity level.

Readiness assignment is matrix-informed but not based on exhaustive matrix-by-matrix scoring. Where matrix coverage or evidential support is incomplete, readiness is assigned conservatively.

B.5 Relationship to reporting and limitations

Readiness describes capability maturity. It does not represent complete method validation or comprehensive performance characterisation.

B.6 Use in this deliverable

At the reporting point for this deliverable, readiness varies across the panel and remains scope-bound and matrix-dependent. Target-specific readiness assignments are provided in Section 5, especially Sections 5.4 and 5.5. Supporting analytical evidence is summarised in Annex C.

In this first iteration, the reported R-levels should be understood as target-level, matrix-informed judgements rather than exhaustive matrix-by-matrix scores.

C ANNEX C - SUMMARY OF ANALYTICAL EVIDENCE

Purpose of this annex

This annex summarises the types of analytical evidence used within the EMBRACE framework to support qualitative detection and identification of biotoxins. It complements Section 4 by presenting a structured overview of evidential elements, rather than a detailed method review, and supports Sections 4.4 and 4.5 by making explicit how qualitative analytical decisions are based on combinations of independent evidence types.

The annex also captures the current maturity of EMBRACE analytical components and tiered workflows (draft, pilot, or defined-scope confirmation-ready status), together with the main internal assets already generated, without implying that all targets have undergone full end-to-end validation.

C.1 Evidence categories and general expectations

Qualitative identification within EMBRACE is based on combined evidence, not on a single analytical signal. The available evidence types are summarised below.

Table C.1. General categories of analytical evidence

Evidence category	Typical examples	Primary role in interpretation
Chromatographic evidence	Retention time (RT), relative retention time (RRT), HILIC vs RP behaviour	Supports selectivity and comparison to reference
Mass-spectrometric evidence (LMW)	Accurate mass, adduct pattern, diagnostic fragments, ion ratios	Supports structural identification of small molecules
Immunological evidence	ELISA signal, antibody capture/detection response	Supports screening and selective recognition
Functional evidence	Endopep cleavage, depurination/adenine release, PP2A inhibition	Demonstrates biological activity
Peptide-level MS evidence	Proteotypic peptides, MS/MS spectra, discriminating peptide logic	Supports structural confirmation of protein toxins
Reference comparison	Reference standards, spectral libraries, spiked controls	Anchors qualitative identification
QA/QC support	Blanks, matrix controls, fortified controls, run acceptance	Validates interpretability of results

General expectations for interpretation are as follows:

- Analytical evidence should always be evaluated in the context of appropriate blanks and controls.

- Where applicable, reference standards or reference spectra should be used for comparison.
- Confirmatory reporting should be based on concordant evidence from more than one analytical dimension where required by the toxin class.
- For LC–MS methods, relevant criteria may include retention behaviour, precursor/product-ion agreement, ion-ratio consistency, and mass accuracy within the defined tolerance window.
- For immunological and functional methods, interpretation depends on response relative to controls or thresholds, specificity of response, and compatibility with expected toxin behaviour.
- Where evidence is incomplete, contradictory, or compromised by control failure, the correct outcome is an inconclusive or not interpretable classification rather than a forced binary conclusion.

C.2 Minimum evidence record fields

To support traceable and reviewable qualitative decisions, EMBRACE uses a minimum evidence record for both low-molecular-weight and protein-toxin methods. The purpose is not to create a fully public spectral library in the current iteration, but to ensure that the key qualitative evidence can be archived, linked to the applied method, and reviewed later if needed.

Table C.2. Minimum evidence record fields for low-molecular-weight toxin MS methods

Field	Required?	Notes
Spectrum or evidence record ID	Yes	Unique and linkable to the sample and run record
Analyte name + identifier	Yes	Use controlled vocabularies where available
Matrix	Yes	e.g. water, shellfish extract, wipe extract, food homogenate
Sample-preparation method ID	Yes	Reference the applied draft workflow or ROP identifier
Instrument + acquisition mode	Yes	e.g. HRMS, QqQ MS/MS, DDA, DIA, PRM
LC method ID	Yes	HILIC or RP route and relevant method identifier
RT, RRT and acceptance window	Yes	Retention behaviour is a primary qualitative criterion
Precursor m/z, adduct, charge state	Yes	Include the preferred evidential form used for review
Key fragments / transitions	Yes	Diagnostic ions or MRM/PRM evidence used for interpretation
Mass error (ppm)	Yes	Define against the method-specific tolerance
Collision energy / settings	Recommended	Useful for reproducibility and method transfer
Reference basis	Recommended	In-run standard, internal library, or literature-backed reference

Field	Required?	Notes
QC status	Yes	Pass/fail plus any relevant flags or interpretive caveats

Table C.3. Minimum evidence record fields for protein-toxin peptide evidence (MS)

Field	Required?	Notes
Peptide ID	Yes	Unique within the EMBRACE evidence register
Parent protein/toxin	Yes	Include isoform or homolog-discrimination notes where relevant
Sequence	Yes	Store securely if needed
Charge states	Recommended	Include the states used for review and confirmation
Expected RT (method-specific)	Recommended	Method-specific and matrix-aware where applicable
Diagnostic ions / fragments	Recommended	Fragments or transitions supporting peptide assignment
Discriminating rationale	Yes	Especially important for ricin vs RCA120-type differentiation
Reference basis	Recommended	Reference material, empirical library, or curated internal library
QC notes	Yes	Capture digestion, enrichment, blank, or recovery dependencies

C.3 Target-specific evidence summaries

The tables below summarise how the EMBRACE toxin classes are currently supported analytically, what the principal confirmatory route is, which orthogonal evidence types are available, and what the current maturity boundary looks like.

Table C.5. Target-specific evidence summary for low-molecular-weight toxin classes

Toxin class	Screening evidence	Confirmatory evidence	Orthogonal evidence	Internal/reference assets	Current readiness	Main current limitation
Saxitoxin/PSTs	LC–HRMS (HILIC); optional immunological screening	HILIC-LC–MS/MS or LC–HRMS with diagnostic fragments and reference comparison	Oxidation-based LC–FLD or derivatisation-based RP-LC	Internal $HRMS^2$ spectra for STX/NEO/GTX analogues; certified standards for selected congeners	R3 (defined scope)	High polarity, matrix dependence, and incomplete congener coverage outside best-characterised matrices
Microcystins and nodularin	Adda-ELISA or equivalent class-based signal	RP-LC–MS/MS or LC–HRMS for defined congeners	MMPB oxidation for total toxin; PP2A inhibition assay	Internal $HRMS^2$ spectra; certified reference standards for MC-LR, -YR, RR and nodularin-R	R1–R2 depending on scope	Congener diversity and the distinction between free and total toxin
Aconitum alkaloids and strychnine-type alkaloids	LC–HRMS target/suspect signal	RP-LC–MS/MS with precursor/product-ion agreement	$HRMS^2$ spectral confirmation; alternative chromatographic behaviour where needed	Internal $HRMS^2$ spectra; reference standards for aconitine and strychnine	R2	Matrix robustness, panel completeness, and finalisation of defined confirmatory scope
Trichothecenes	LC–HRMS target/suspect detection	RP-LC–MS/MS for known analytes	HRMS-based suspect screening; derivatisation or class-fragment logic	Internal $HRMS^2$ spectra; Standards for T-2, HT-2, and selected congeners	R2	Modified forms, analyte diversity, and matrix-dependent behaviour

Selected representative data examples are included below to showcase internal evidence. They are illustrative only and should be interpreted together with the evidence tables, reference comparison, and QA/QC logic rather than as stand-alone proof.

Figure C.1. Representative HILIC–MRM chromatograms for selected PST standards (100 ppb spike in urine). Method summary: BEH-AMIDE 2.1 × 100 mm; gradient 98%→50% A in 7 min, hold at 50% A for 2 min, then to 0% A in 2 min; A = ACN/H_2O (7:3) + 0.1% FA; B = 8 mM ammonium formate + 0.1% FA; four MRM transitions, optimised CID, dwell 6.3 ms. Included as a representative chromatographic demonstration rather than a claim of full cross-matrix validation.

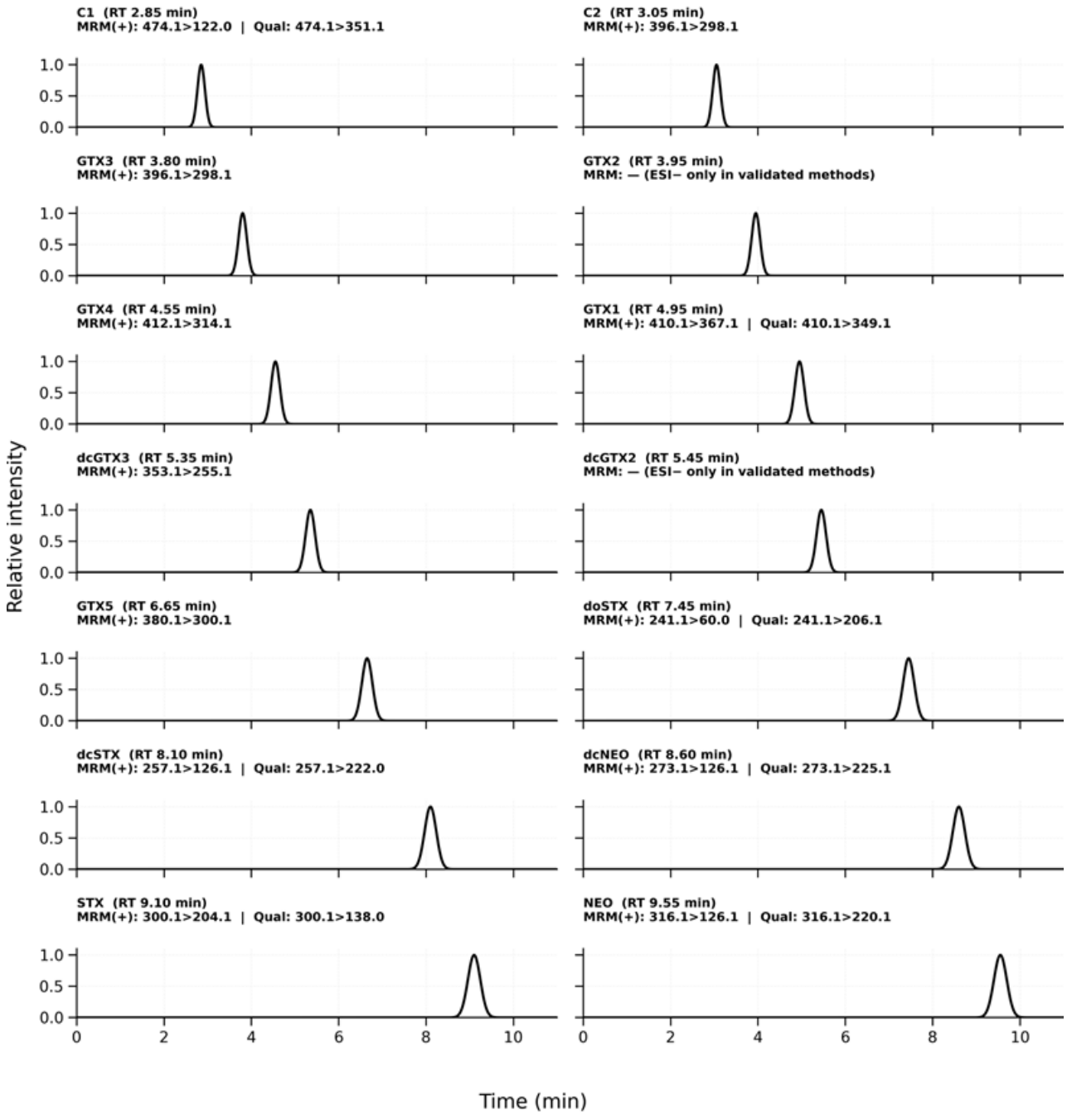


Table C.6. Target-specific evidence summary for protein toxins

Toxin class	Screening evidence	Confirmatory evidence	Orthogonal evidence	Internal/reference assets	Current readiness	Main current limitation
Ricin	Sandwich ELISA with dual antibody recognition of A- and B-chain targets	Peptide-level LC–MS/MS after selective enrichment and tryptic digestion	Depurination / adenine-release LC–MS/MS; orthogonal immunological support	EMBRACE ricin peptide HRMS/MS dataset, ELISA assay, adenine-release workflow, ricin reference materials, and antibody resources	R3 (defined scope)	Dependence on enrichment reagents, digestion efficiency, and extension of robustness across additional matrices
Botulinum neurotoxins	Immunological detection and immunocapture	Functional Endopep assay with peptide-level confirmation logic under development	Multiple Endopep readouts (SIA, MALDI-TOF, LC–MS/HRMS) based on cleavage products	Reference materials, serotype-specific capture antibodies, neoepitope antibodies, and draft assay protocols	R1	Workflow still under development; matrix dependence, reagent dependence, and incomplete consolidation of the full evidential chain

C.4 Example of tiered interpretive wording for the most mature workflow

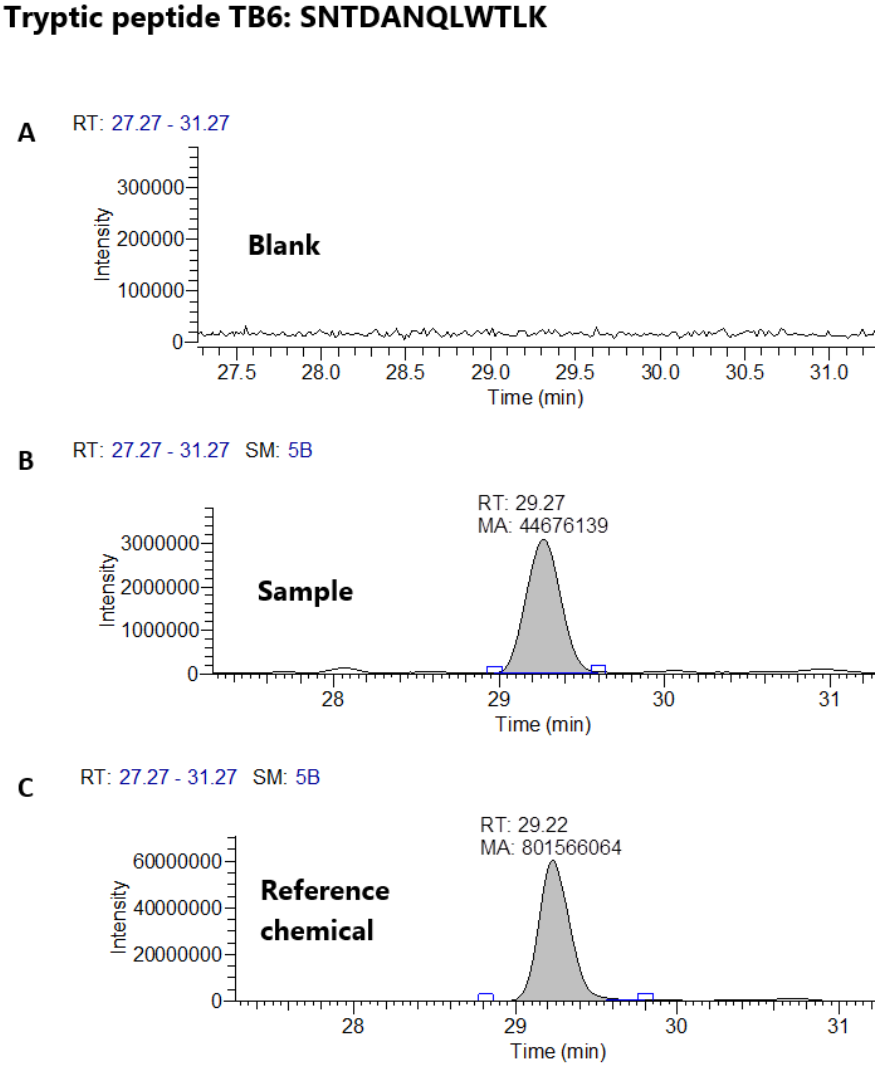
Ricin is currently the most advanced EMBRACE protein-toxin example. The wording below illustrates increasing evidential strength.

Table C.7. Example tiered wording for ricin

Tier	Illustrative wording
Presumptive (Tier 1)	Immunoassay indicates a presumptive ricin-family positive. Controls met acceptance criteria. Due to known immunological cross-reactivity potential, confirmatory peptide LC–MS/MS is required for a confirmed identity statement.
Probable (Tier 2)	Peptide MS results indicate a probable ricin identity based on at least two proteotypic peptides, but the reference comparison requirement is not fully met (for example, no in-run reference material or incomplete discriminating peptide coverage).
Confirmed (Tier 3)	Ricin confirmed by peptide LC–MS/MS through detection of discriminating proteotypic peptides with MS/MS agreement to reference material or curated library, with all QC criteria satisfied.
Confirmed + activity (Tier 4)	Ricin confirmed (Tier 3) and functional depurination evidence indicates active toxin consistent with ricin mechanism.
Inconclusive / Not interpretable	If peptide MS is compromised by failed digestion controls, blank contamination, or insufficient recovery, classify as not interpretable (QC failure). If screening is positive but peptide MS and functional results are negative with passing QC, classify as inconclusive and describe the evidence conflict.

A representative ricin confirmation example is shown below to illustrate how blank comparison, sample signal, and in-run reference material can be presented for the peptide-identification layer.

Figure C.2. Representative peptide-level ricin confirmation example. Extracted ion chromatograms for ricin tryptic peptide SNTDANQLWTLK (TB6) show absence of signal in the blank and concordant retention with the ricin reference material. FTMS2 spectrum of m/z 695.8 @ peak 29.2 min shown in **fig C.3** identifies the peak as SNTDANQLWTLK (TB6). This is illustrative of the peptide-identification layer and should be interpreted together with enrichment, digestion, reference comparison, and QC acceptance.



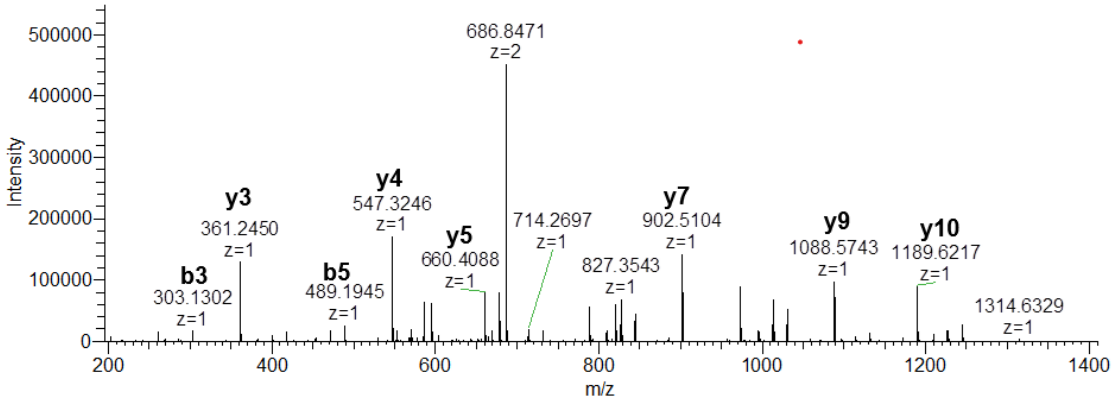
NanoLC–HRMS2: EIC of 695.8^{2+} precursor ion, full ms^2 scan

A= Solvent blank

B= Sample chemical at 29.27 min.

C= Reference chemical ricin at 29.22 min

Figure C.3. Annotated FTMS2 spectrum of m/z 695.8 @ peak 29.2 min shown in fig C.2B.



MS/MS spectrum of the peptide TB6 (m/z 695.82^{2+}). The peptide sequence (SNTDANQLWTLK) is annotated with the detected fragment ions.

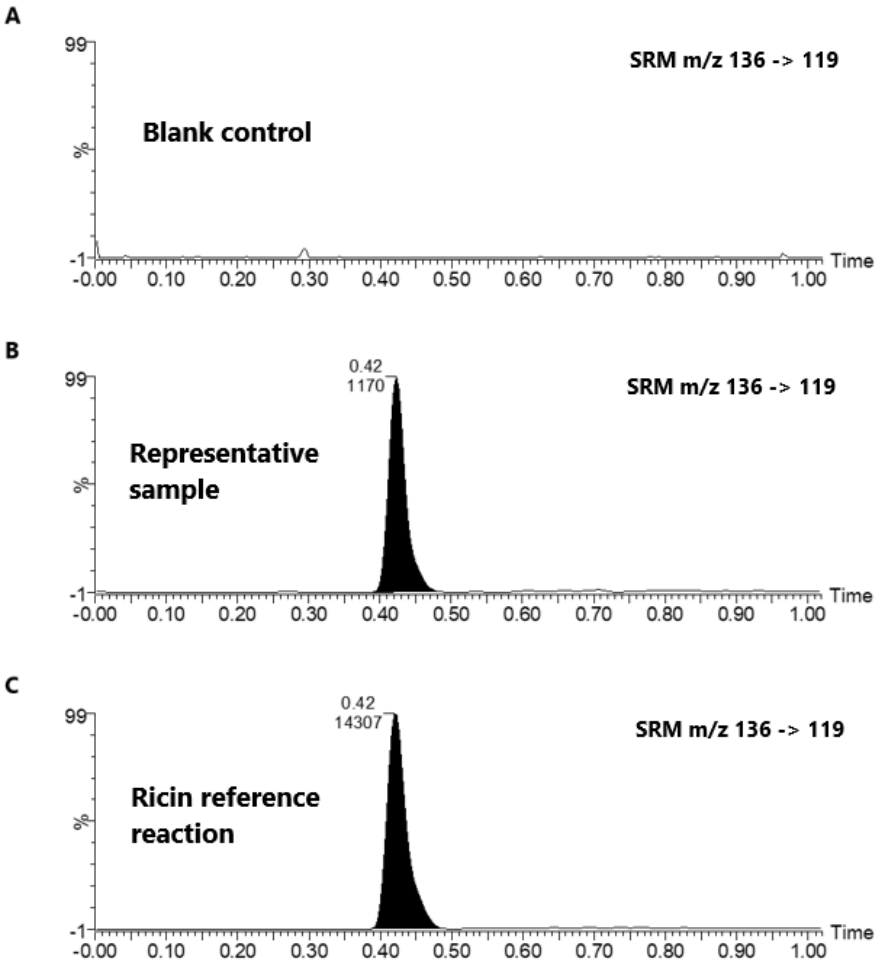
Theoretical m/z	**Observed m/z**	**Error (ppm)**	**b ions**	**Peptide seq**	**y ions**	**Theoretical m/z**	**Observed m/z**	**Error (ppm)**
695.8517	**695.8516**	**0.14**						
				S				
202.0822			b2	**N**	y11	1303.6641		
303.1299	**303.1302**	**0.99**	b3	**T**	y10	1189.6212	**1189.6217**	**0.42**
418.1569			b4	**D**	y9	1088.5735	**1088.5743**	**0.73**
489.194	**489.1945**	**1.02**	b5	**A**	y8	973.5465		
603.2369			b6	**N**	y7	902.5094	**902.5104**	**1.11**
731.2955			b7	**Q**	y6	788.4665		
844.3795			b8	**L**	y5	660.4079	**660.4088**	**1.36**
1030.4588			b9	**W**	y4	547.3239	**547.3246**	**1.28**
1131.5065			b10	**T**	y3	361.2445	**361.245**	**1.38**
1244.5906			b11	**L**	y2	260.1969		
				K	y1			

Theoretical and observed sequence ions for the SNTDANQLWTLK peptide.

Figure C.4. Ricin activity assay. LC–ESI–MS/MS chromatograms of released adenine (m/z 136 → 119) for blank, sample, and ricin reference. The sample shows a peak at ~0.42 min matching the reference in retention time and ion ratio, with no signal in the blank, confirming adenine release consistent with ricin activity. Quantifier (136 → 119) and qualifier (136 → 92) transitions were monitored.

Ions/transitions	Sample		Standard		Criteria	Result
	Area*	Ion Ratio	Area*	Ion Ratio	Tolerance#	
136 → 92 (q)	543		6821			
136 → 119 (Q)	1170	46.4%	14307	47.7%	±11.9%	ID

* Peak area expressed as relative intensity (%) of the base peak.
# Ion ratio tolerances relative to the reference standard: 50%: ±20%; >20–50%: ±25%; >10–20%: ±30%; ≤10%: ±50%.



C.5 Readiness snapshot across current EMBRACE targets

The current iteration is intentionally heterogeneous in maturity. Readiness should therefore be interpreted as defined-scope and matrix-sensitive rather than as a universal claim of transferability across all matrices and all laboratories.

Table C.8. Current readiness snapshot and main gap

Target / toxin class	Identification readiness	Attribution-oriented status	Main current gap
Botulinum neurotoxins	R1	Preparatory only	Serotype-specific implementation; consolidation of the full evidential chain and confirmation scope
Ricin	R3 (defined scope)	Preparatory only	Extension of robustness and clearer matrix/scope boundaries
PSTs / saxitoxin-group toxins	R3 (defined scope)	Not yet established	Extension beyond best-characterised matrices and broader forensic scope
Aconitum alkaloids	R2	Not yet established	Panel completion, matrix robustness, and confirmatory decision logic
Strychnine-type alkaloids	R2	Not yet established	Matrix robustness and integration into final decision/reporting framework
Trichothecenes	R2	Not yet established	Matrix complexity, modified forms, and final confirmatory scope
Microcystins and nodularin	R1–R2	Not yet established	Progression from broad screening toward clearly defined confirmatory scope

C.6 Representative matrices covered in current iteration

Current analytical readiness is matrix-dependent. The matrices listed below represent those addressed in method-development work or internal illustrative datasets. The table reflects current practical scope and does not constitute a validation summary.

Table C.9. Current practical matrix scope represented in method-development work or internal illustrative datasets

Matrix class	Representative matrix	Toxin classes currently represented	Current scope note
Liquid / simple	Saline or buffer	PSTs, aconitine, strychnine, MC-LR, T-2 toxin, ricin	Reference standards, spike solutions, and simple liquid conditions used for primary optimisation and reference comparison.
Liquid / complex	Beverages / drink-like liquids	PSTs, aconitine, strychnine, MC-LR, T-2 toxin, ricin	Extraction pilot datasets; transfer beyond simple solvent conditions.
Biofluid	Urine	PSTs, aconitine, strychnine, MC-LR, T-2 toxin	Pilot complex matrix and chromatographic tests; a representative PST urine dataset is shown in Figure D.1.
Biofluid	Serum / plasma	Aconitine, strychnine, T-2 toxin; ricin in serum	LMW work remains at pilot scope; ricin serum matrix has also been used in enrichment, recovery, and selectivity work.
Solid / food	Mussel / shellfish	PSTs	Canonical PST food matrix and an important extract class for congener-resolved confirmation workflows.
Solid / environmental	Soil / sand	Ricin; PSTs at exploratory scope	Ricin complex solid matrix has been exercised directly.
Solid / biological	Cat feces	Ricin	Complex biological solid matrix exercised in ricin enrichment work.
Solid / food	Chocolate / foodstuffs	PSTs	Rich food-matrix pilot; not a broad cross-matrix validation.

C.7 Supporting analytical assets generated in the first iteration

Table C.10. Supporting analytical assets already available or drafted

Asset type	Current content	Role in qualitative evidence
Internal reference spectra	$HRMS^2$ reference spectra generated for STX and analogues, microcystins/nodularin, plant alkaloids, trichothecenes and ricin tryptic peptides	Support spectral matching, fragment review, and internal reference comparison
Reference materials and standards	Network or certified materials for BoNT/A, B, ricin D/E, selected PST congeners, MC-LR, nodularin-R, T-2, HT-2, aconitine-group alkaloids, and strychnine	Provide reference comparison, method development support, and defined-scope confirmation anchors
Antibody resources	Ricin ELISA antibodies and BoNT capture / neoepitope antibodies for Endopep and immunodetection modules	Enable screening, selective enrichment, and orthogonal protein-toxin evidence
Draft analytical workflows	Harmonised LC–MS screening workflow for selected low-molecular-weight toxins; draft Endopep procedures; draft ricin adenine-release LC–MS/MS; draft sandwich ELISA procedures	Define intended analytical logic, control concepts, and decision points in operational form
Analyte tables and decision-support elements	Preliminary analyte lists, precursor ions, candidate product ions/transitions, adduct forms, diagnostic fragments, peptide targets, and identification criteria	Bridge strategy-level design and analyte-level interpretation

C.8 Current limitations

Several limitations remain important to the interpretation of current analytical evidence and should be stated explicitly rather than treated as exceptional conditions.

- Matrix-dependent signal suppression, interferences, or enrichment losses remain a dominant cross-cutting constraint.
- Reference standards, spectral libraries, and critical reagents are still unevenly available across the toxin panel.
- Protein-toxin workflows depend strongly on enrichment reagents and sample-preparation performance.
- Modified or transformed analytes are not yet covered comprehensively for all toxin classes.
- Current readiness reflects primarily in-house development within one laboratory and should not be interpreted as interlaboratory validation.
- Attribution-oriented analysis remains a separate and less mature development line than core detection and identification.

C.9 Summary

The first iteration comprises structured evidence tables, defined interpretive logic, a matrix-scope summary, and selected representative data figures. Identification is based on the combination of

independent evidence types, supported by internal reference assets and constrained by defined scope and current maturity.