Challenges in the Development of Reference Materials for Protein Toxins

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High molecular weight protein toxins produced by bacteria, *e.g.* staphylococcal enterotoxins and botulinum neurotoxins, as well as plant toxins such as ricin and abrin, are relevant analytes in different application areas: food safety, public health, civil security and defense sector, and – in case of botulinum neurotoxins – also in pharmaceutics. For their reliable and accurate detection, identification and quantification, reference materials (RMs), in particular certified reference material (CRM), are required. The present article focuses on challenges in the development (production and certification) of such RMs. Firstly, it highlights the role of RMs and CRMs, what they can be used for, the nature of certified properties, metrological traceability, and uncertainty of certified values, as well as commutability of RMs. Secondly, the molecule-specific technical challenges are highlighted using the example of the mentioned toxins. This includes for instance the choice of a suitable purification strategy (recombinant expression and purification versus the purification of toxin from natural sources), the in-depth characterization of the obtained preparations by a comprehensive set of methods including immunochemical assays, mass spectrometry, and functional assays to verify their identity and establish their purity and activity, and finally, suitable approaches for determining reference values of important toxin properties (protein mass concentration in solution, biological activity). The article summarizes

ongoing activities in a new European initiative called EuroBioTox, which aims at the production and certification of RMs for selected protein toxins and the establishment of validated procedures for the detection and identification of biological toxins.

Biological Toxins

Biological toxins are a large group of hazardous substances produced by living organisms such as bacteria, plants, or animals, which exert a detrimental effect on other organisms upon uptake. Among those, high molecular weight protein toxins deserve special attention since they are relevant in different fields: Bacterial toxins such as staphylococcal enterotoxins (SE) or botulinum neurotoxins (BoNT) are known as causative agents of food poisoning outbreaks and are therefore monitored by national and international food and health agencies [1-3]. On the other hand, the same toxins have been linked to military research programs in the past where staphylococcal enterotoxin B (SEB) has been explored as incapacitating agent and BoNT has been weaponized under the code name "X" [4-6]. Along the same line, the plant toxins ricin or abrin have long been known to induce natural intoxications in humans and animals [7-8]. Ricin, similar to BoNT, has a history of military research (code name "W") and is the only protein toxin listed in Schedule 1 of the Chemical Weapons Convention[9]. Based on the potential threat level in public health incidents, BoNT as well as ricin and SE have been classified as bioterrorism agents of the highest (BoNT) or second highest (ricin, SE) category [10-11].

Recent incidents in Europe and worldwide have threatened civil society by the attempted use of different biological toxins. Exemplarily, in June 2018 a biological terror attack was thwarted in Cologne, Germany, where the suspect was accused of having manufactured ricin and acquired bomb-making materials for a serious act of violence against the state [12]. Therefore, increased vigilance and adequate preparation is of importance in a world facing growing risks of man-made disasters.

From an analytical perspective, protein toxins have several commonalities even though they are produced by different organisms and have quite different structures and functions [13]:

1) They are toxic in the absence of the producing organism and its genetic information. Therefore, detection has to focus on protein-based methods, DNA-based methods are not sufficient in most instances.
2) Their very high toxicity demands for highly sensitive methods, optimally with detection limits in the pg/mL range when different sample types are analyzed.

3) They are rapidly metabolized after uptake in the human body, limiting the time window for detection in clinical specimens.
4) Finally and most challenging, they are often produced in multiple variants or isoforms which might differ in their toxicokinetics and toxicodynamics.

Detailed information on the individual toxins, their biological structure and function has been reviewed elsewhere (for an overview please see [13-16]). Briefly, for the detection of biological toxins, a variety of methods has been established based on immunological, spectrometric, and functional assays or combinations thereof – all of those have advantages and limitations. Among those, immunoassays such as sandwich enzyme-linked immunosorbent assays (ELISA) display the presence of the analyte and highlight the native folding of the molecule depending on the antibodies used [13, 17-19]. Among all technologies available, ELISA-based methods still provide the highest sensitivity with detection limits in the ng/mL to fg/mL range provided that high-affinity antibodies are used [18-19]. However, for ELISA-based methods, the recognition of individual toxin variants has to be comprehensively tested, and the discrimination of closely related toxin variants is not always feasible [20-22]. In this context, mass spectrometry methods (*e.g.,* matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF-MS) and liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI MS)) are able to deliver unambiguous sequence information and therefore provide reliable identification of toxins. With modern instrumentation, sensitivities are still somewhat limited and can reach down to a few ng/mL of toxin, especially when combinations of immunoaffinity-based enrichment, tryptic digestion plus MS-based detection and identification of specific peptides is applied [18-19, 23-24]. Finally, functional methods display the biological activity or potency of protein toxins. Here, many different approaches have been described for the individual toxins ranging from *in vivo* assays (*e.g.,* mouse bioassay for BoNT), *ex vivo* assays using animal tissues (*e.g.,* mouse phrenic nerve hemidiaphragm assay for BoNT) or *in vitro* assays which renounce te use of animal tissues [25-27]. Depending on the protein toxin, functional *in vitro* assays display receptor binding, internalization and enzymatic activity or only parts thereof (*e.g.*, cytotoxicity assay versus adenine release assay for ricin and abrin; endopeptidase assay for BoNT [28-30]) or they display the interaction of toxins with their physiological targets (*e.g.*, TCR / MHC-binding in a mixed lymphocyte reaction for SE [31]).

In the past, an objective comparison of the different methods has not been possible since no suitable RM and no opportunity for proficiency testing has been available. In light of the relevance of biological toxins in the food, health and security sectors and in order to establish the *status quo* of detection capabilities within the European Union (EU) and beyond, a project called EQuATox was funded by the EU's 7th framework program from 2012 to 2014 (EQuATox,

"Establishment of Quality Assurances for the Detection of Biological Toxins of Potential Bioterrorism Risk") [1, 32-33]. Here, the evaluation of technical capabilities in a series of proficiency tests (PTs) showed that mostly satisfactory results were obtained in international expert laboratories when dealing with basic analytical tasks; still the equivalence of analytical results would clearly profit from further technical improvement [1, 13, 18-19, 34]. Among other findings, it became clear that expert laboratories use indeed a broad panel of different tools and technical approaches for detection, identification and quantification of biological toxins resulting in data of variable quality. Though validation studies were published for individual methods, there are currently hardly any agreed-upon reference methods available, nor are there RMs available for the toxins in focus (ricin, abrin, SE, BoNT). So expert laboratories currently use either own in-house purified materials or commercial toxins of varying quality as quality-control (QC) samples or for calibration, which makes any comparison of different methods applied in different laboratories questionable [13].

As an outcome of the project, a roadmap for harmonization of detection methods for biological toxins was drafted [13] which is now implemented in the ongoing EuroBioTox project funded by the EU's Horizon 2020 program from 2017 to 2022 ("European Programme for the Establishment of Validated Procedures for the Detection and Identification of Biological Toxins") [35-36]. In this project, 13 core members join their forces together with 48 network partners from 23 countries to work on a comprehensive package of quality assurance measures, including the production and certified reference materials (CRMs), the refinement of analytical procedures, the availability of tools in a European repository, state-of-the-art training on good analytical strategies, and the establishment of a comprehensive proficiency testing scheme. A major focus of the project is the production of CRMs for prioritized biological toxins such as ricin, BoNT and SEB.

On the Importance of Quality-Control Tools

In case of a natural (*i.e.*, food poisoning outbreak) or intentional release (*i.e.,* bioterrorism threat scenario) of biological toxins, decision makers rely on correct and reliable laboratory data to make appropriate and timely decisions, to manage the threat and to alleviate the outcomes on society. In this context, a high level of analytical capability is required to understand the scale of the incident and to take qualified decisions on countermeasures [13]. Generally, several components are needed in order to perform reliable measurements:

- Good analytical strategies

- Validated methods for screening, identification, quantification and measurement of biological activity; the latter allows for the determination of the biological threat level
- RMs, preferably certified RMs (ideally both pure toxin solutions ("calibrants") as well as toxin in matrix materials)
- Standard operating procedures
- Regular training of personnel
- External evaluation of measurement capabilities by proficiency testing
- Continuous refinement of methods and development of innovative/superior methods

The use of QC tools properly characterized with respect to identity and purity is an important requirement to achieve accurate and reliable results in the measurement of biological toxins [37]. By definition, ISO Guide 30 defines an RM as a material which is sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process [38]. The property is the entity for which a reference value is established. This property can be qualitative (*e.g.,* identity of species: genomic DNA of *Listeria monocytogenes, strain 4B, NCTC 11994)*, but in most cases it is quantitative (*e.g.,* mass concentration of a given protein in a matrix: amyloid β1-42 peptide in human cerebrospinal fluid, 0.72 ± 0.11 µg/L [examples from JRC RM catalog [39], CRMs IRMM-449 and ERM-DA481/IFCC, respectively]). A special category are RMs used for presence/absence testing, where the property is typically expressed as probability of detection (*e.g.*, *Staphylococcus aureus* enterotoxin A (SEA) in cheese, CRM IRMM-359) [40].

A **certified reference material (CRM)** is an RM characterized by a metrologically valid procedure for one or more specified properties, accompanied by an RM certificate that provides the value of the specified property (the certified value), its associated uncertainty, and a statement of metrological traceability [38]. The associated uncertainty is expressed as so-called expanded uncertainty, which has the meaning of a confidence interval in which the true value lies with a certain probability, typically 95%. The qualification requirements for CRM producers are laid down in ISO 17034 ("General requirements for the competence of reference material producers")[41], a standard which recently has been published after its conversion from the former ISO Guide 34[42]. The Reference Materials Unit within JRC Geel (formerly Institute for Reference Materials and Measurements, IRMM) obtained accreditation to ISO Guide 34 in 2004, and is meanwhile accredited according to ISO 17034. Whenever possible, RM production (processing and certification) projects are carried out under this accreditation. In addition, all finalized projects undergo review by subject-specific external expert panels before respective materials are released for sales. Metrological traceability is a key concept of metrology and is strongly connected with uncertainty. Metrological traceability is defined as "property of a measurement result whereby

the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty" [43-44]. The practical meaning is that if a valid metrological traceability chain is established, the measurement result in a laboratory using its in-house routine method and analyzing a routine sample can be linked to a metrological reference as anchor point, which can be the practical realization of an SI unit (*e.g.,* sample mass is 5.02 kg), a measurement procedure (*e.g.,* amyloid β1-42 peptide as obtained by solid phase extraction and subsequent quantification by liquid chromatography with mass spectrometry detection, according to the reference methods [45-46]), or an artifact (*e.g*., 1 international unit (IU) is equivalent to 0.0347 mg of human insulin [47]). Metrological traceability is essential to make results comparable over time and space, with profound consequences, *e.g.*, in pharmaceutical products and in international trade (import and export of goods). Metrological traceability is undoubtedly important for measurement results, but equally important for certified values of a CRM. Establishment of metrological traceability is accomplished by using existing CRMs of qualified National Metrology Institutes (NMIs) or other institutions of equivalently demonstrated competence. In this specific case, no such CRMs are available, which rules out this possibility. Another means, however, is a demonstration of the RM producer's competence, *e.g.,* through participation in key comparisons under the umbrella of the CIPM-MRA (Mutual Recognition Arrangement under the Comité International des Poids et Mesures/International Committee for Weights and Measures). JRC Geel (formerly IRMM) has participated in various studies organized in dedicated CCQM (Consultative Committee for Amount of Substance: Metrology in Chemisty and Biology) working groups (*e.g.,* Bioanalysis Working Group, recently further split down into Protein Analysis[48], DNA Analysis, and Cell Analysis Working Groups; Organic Analysis Working Group, Inorganic Analysis Working Group). The intercomparisons among NMIs serve as benchmark and aim at demonstrating the degree of equivalence of results obtained by the participants.

Another important metrological concept shall be briefly mentioned: commutability of a CRM. It shall be understood as the degree of equivalence in the analytical behavior of real samples and a CRM with respect to various measurement procedures (methods). The term commutability originated from the clinical chemistry field [49]. It describes the ability of an RM to have inter-assay properties comparable to the properties demonstrated by authentic clinical samples when measured by more than one analytical method [50]. Preparation of the CRM (formulation procedures as lyophilization, additives for preservation) can potentially lead to non-commutability of the material, for instance due to alteration of the analyte and/or changes in the matrix which impairs extraction efficiency of the analyte.

Investigation of suitable commutability is especially important for matrix CRMs. Again, various examples can be listed from the clinical RM sector where dedicated commutability studies aim to demonstrate that the RM investigated behaves in the same or comparable way to patient samples [51].

The certificate of a CRM describes the material, indicates the property value (certified value), its corresponding uncertainty, in case of method-dependent measurands the method(s) that was (were) used to obtain the data contributing to establish the certified value and its uncertainty, the reference to which the certified value is traceable, and the name and function of the RM producer's approving officer [52]. Moreover, other information typically present on CRM certificates include safety information, instructions for storage, how and what to use the material for, reconstitution protocol, if applicable, and a legal disclaimer.

The typical uses of CRMs comprise the following applications:

- Calibration of a method. Typically, **calibrants** are pure substances or solutions of a pure substance (a small molecule, but also biomolecules such as a protein). There are some exceptions, especially in the clinical field, where **matrix CRMs** – a calibrant spiked in defined amount into a given matrix – are used for calibration: If an isolated pure protein would behave differently in a measurement process compared to a sample where that protein is in its natural matrix (*e.g.,* human serum), matrix CRMs are recommended for method calibration.
- Validation of methods. For assessing the trueness of a method, a matrix CRM is indispensable since all extraction steps are included in the analytical workflow.
- Method performance verification. In this context, the CRM is used as QC sample. The aim is to demonstrate that when applying the method, the certified value is found, thus the method performs correctly.

It is important to note that for each CRM it has to be defined case-by-case for which purpose and application it can be used. For instance, a calibrant solution can be used in method validation studies, but not for the parameter trueness (matrix is absent). As mentioned earlier, a matrix CRM on the other hand is usually not used for calibration of a method.

Development of RMs and CRMs

The development of RMs and especially CRMs is a complex process. Exemplarily, Figure 1 displays the general outline of a CRM production at JRC Geel.

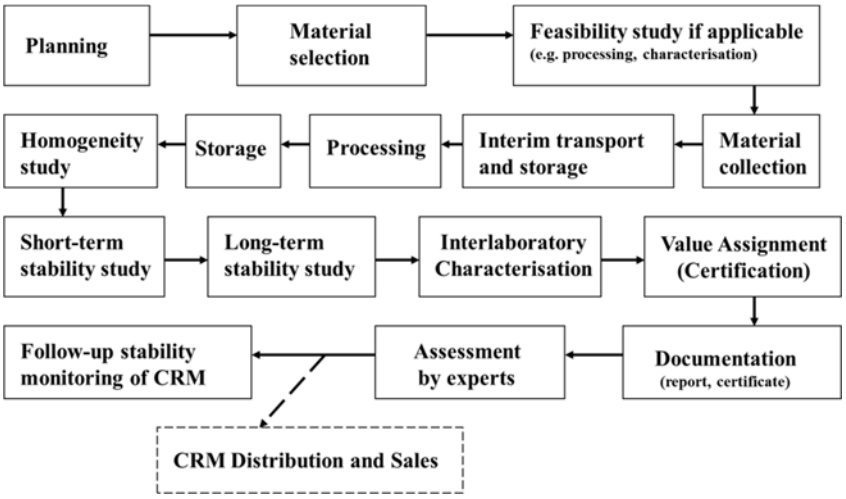

Figure 1. General procedure for CRM production applied at JRC Geel. [53]

Material selection can sometimes be difficult, especially for some matrix RMs (*e.g.,* decision on exact matrix or accessibility of suitable raw materials). Often orienting feasibility studies are required, for instance to investigate how processing of a suitable RM can be performed. Processing usually is a multi-step process, *i.e.* to convert a raw material such as plant seeds containing a biological toxin into an RM preparation, here a purified toxin isoform. Processing typically contains several steps. It can comprise but is not limited to extraction, purification by means of preparative chromatography (different modes), formulation, aliquoting into containers, and lyophilization. Once the so-called candidate RM batch is produced, it is aliquoted into the final storage containers (*e.g.,* plastic vials), ensuring that no filling trends occur over the large number of containers filled.

Homogeneity is a key requirement for any RM aliquoted into units. To demonstrate suitable homogeneity, a study is carried out to verify equivalence between the units produced and to quantify the between-unit variation [54]. **Stability**

testing is necessary to establish the conditions for storage (long-term stability) as well as the conditions for dispatch of the materials to the customers (short-term stability). Again, dedicated studies are executed to demonstrate at which temperatures the RM (CRM) can be safely stored (*e.g.,* -80 °C) and how shipments have to be executed (*e.g.*, dry ice shipment to customer). Whenever possible, so-called isochronous stability studies are the primary choice, as measurements will be carried out in a large series, thereby adhering to repeatability conditions and thus minimizing the variability that stems from measurements [55].

One central aspect as concerns measurements within an RM (CRM) project is the availability and accessibility of high-performing methods, *i.e.* methods to be used for homogeneity and stability measurements (key parameters precision and intermediate precision) and essentially for characterization (key parameters trueness, precision and intermediate precision). Characterization of RMs is defined as determination of the property values or attributes of an RM, as part of the production process. These property values (*e.g.,* protein mass concentration of toxin X as established by method Y is 1.52 mg/mL) once established are then called **certified values** when the RM project is completed and certificates have been established (value, assigned to a property of an RM that is accompanied by an uncertainty statement and a statement of metrological traceability, identified as such in the RM certificate [38]).

Strictly speaking, validation of methods – especially the parameter trueness – require CRMs, and, *vice versa*, the production of CRMs (establishment of certified values for a given property) require validated methods, whereby trueness and precision ("accuracy") is key. This vicious circle or chicken-egg dilemma can only be overcome by a step-wise approach. To this end, it is crucial to understand the methods (measurement processes) to the best possible extent. This comprises but is not limited to comprehensive validation (*e.g*., assessment of specificity, extraction recoveries and matrix effects), use of existing suitable CRMs whenever possible, and regular participation in PT studies to demonstrate the competent application of a method in a laboratory. RMs from JRC Geel come with comprehensive documentation: the RM certificate and a certification report, which outlines the background, production of the material, homogeneity, stability and characterization studies and results, value assignment, uncertainty calculation, metrological traceability statement, list of references, and annexes in most cases containing graphics and charts.

Challenges in the Development of CRMs for Protein Toxins

As mentioned above, the current EU-project EuroBioTox addresses the production and characterization of CRMs for different plant and bacterial protein toxins, among them ricin, BoNT and SEB. Specifically for those high molecular weight toxins, there are a number of practical challenges to deal with.

1) The **purification or production strategy** has to be decided on – shall the CRMs be produced by purification of toxins from their natural sources or shall they be produced by recombinant technologies? Actually, the answer has to be given on a case-by-case basis and is closely linked to the molecular characteristics of the toxin CRM to be produced.

As an example, ricin is a plant toxin with a complex and variable glycosylation pattern containing four glycosylation sites, two on the A and B chains each [8, 56]. Importantly, the type and level of glycosylation affects the functional activity of ricin [20, 57-58]: of differently glycosylated ricin isoforms tested, the highest glycosylated form, containing more hybrid/complex-type glycans, was most toxic in different biological assays tested. Conversely, chemically deglycosylated ricin A turned out to be approximately 1000-fold less toxic than glycosylated ricin A [59]. N-glycosylation was shown to promote the toxicity of the ricin A chain by promoting its transport out of the endoplasmic reticulum [60]. In light of the available information on ricin's functional activity, the authentic glycosylation pattern seems to be crucial for its enzymatic activity. Therefore, production from natural sources is – for the glycoprotein ricin – a superior approach compared to recombinant expression, which most likely would not result in correctly glycosylated toxin. Also, considering the cellular toxicity of full-length ricin, recombinant production would be difficult to pursue in eukaryotic expression systems (which in principle are able to deliver glycosylation). As an alternative production strategy and to circumvent ricin's cytotoxicity, advanced cell-free expression systems could be used which are able to add post-translational modifications; however, the authenticity of the glycosylation pattern compared to the natural plant-derived toxin would have to be proven.

The method of choice for non-glycosylated bacterial toxins such as SE and BoNT is the recombinant production, *e.g.*, in *E. coli,* which ensures reliable expression, high yields and purity following established protocols. Especially the anaerobic cultivation of *Clostridium botulinum* is empiric and error prone. In addition, BoNT is encoded together with up to five neurotoxin-associated proteins (NAPs) such as the non-toxic non-hemagglutin of 140 kDa and three hemagglutinins which form large toxin complexes up to 760 kDa. Isolating the 150 kDa BoNT from these toxin complexes is challenging and requires sophisticated procedures. The drug substances of all currently approved BoNT-based pharmaceuticals are

isolated as toxin complex from *C. botulinum* culture supernatant, but only two drug products (inco- and daxibotulinumtoxin A) comprise just the pure 150 kDa BoNT/A illustrating the challenge to separate BoNT from the NAPs. Recently, the first recombinantly produced BoNT drug product (rBoNT-E) was successfully evaluated in a clinical phase I trial (EudraCT 2016-002609-20) and further recombinantly produced BoNTs are in development as pharmaceuticals. In case of BoNT and SE with their >40 and >26 toxin variants, respectively, it is extremely valuable being able to use codon-optimized synthetic genes. This approach allows the expression of any toxin whose sequence is known or deposited in a public database without the need to obtain the natural producing strain or its genomic DNA. One important prerequisite, however, is that it can be demonstrated in a pre-study that native and recombinant protein behave the same way or at least as similar as possible in different methods. For instance, if the toxin was equipped with an affinity peptide tag to increase the isolation efficiency, the tag removal after purification is important so that the interaction in an immunochemical assay (*e.g.*, ELISA) or in a functional assay is not impaired. In contrast to SEs, BoNTs are classical AB-toxins and require proteolytic activation into a 50 kDa light chain (LC) and a 100 kDa heavy chain (HC) which remain covalently linked by a disulfide bridge for biological activity. Since recombinant expression of BoNTs in *E. coli* yields a single polypeptide chain which is biologically inactive, subsequent specific and quantitative hydrolysis (>95% di-chain BoNT) at a defined loop region between LC and HC is essential to obtain biological activity. This process is best controlled *in vitro* with specific proteases and, if necessary, with an engineered loop sequence. Of course, the used protease, any peptides cut out from the loop as well as affinity tags cleaved off the BoNT need to be quantitatively removed at a rather late stage of the purification process to ensure high purity of the RM. Some BoNT variants like all BoNT/E subtypes are released by group II non-proteolytic *C. botulinum* and hence always occur as single polypeptide chain with low biological activity until being hydrolyzed in the patients' gastrointestinal tract. In contrast, a recombinantly produced BoNT/E including a proteolytic activation step already yields the biological highly active toxin which is suitable as RM for sensitive detection methods. Whereas the biological consequences of the disulfide bond formation in *e.g.* SEB is yet unknown, BoNTs absolutely depend on the intact disulfide bond connecting LC and HC to exert their neurotoxicity. Due to the aerobic conditions during recombinant expression and isolation of the BoNT its formation has been proven for all established BoNT serotypes [61]. Altogether, the 'recombinant way' is highly suitable for the production of SE and BoNT RM.

2) A second important decision in the planning phase of a toxin CRM project is the decision on which **molecular toxin variant** to use. Is it more appropriate to use one isolated toxin variant – and if yes, which one, or would a defined mixture of naturally occurring variants better reflect the analytical task? The large batch

size of CRMs has to be taken into account: can the selected toxin variant be produced in sufficient amount and purity? Generally, a high purity of the analyte (≥ 95%) is desirable in a CRM production process. Lower purity preparations are acceptable if the uncertainty of the certified value is adjusted accordingly with properly estimated contributions from respective impurities. Residual impurities have to be identified and quantified individually whenever possible. This includes not only protein/peptide impurities, but also impurities of other nature, *e.g.* inorganic impurities[62]

Considering the occurrence of plant toxins in multi-gene families, the differentiation of the analyte toxin in the CRM from co-purified residual impurities can be challenging when purifying from natural sources [20]. As an example, ricin occurs in two major isolectins, ricin D and ricin E, which are present in most *R. communis* cultivars and are 97% identical on the protein level [63-65]. Ricin is composed of a cell-binding B subunit and an enzymatically active A subunit, both linked by a single disulfide bond forming a 63 kDa protein [66]. Additionally, the plant expresses a highly related molecule called *Ricinus communis* agglutinin (RCA120) which is a tetrameric protein of two ricin A-like and two ricin B-like subunits. The identity on the protein level between the A and B chains of ricin D to those of the related RCA120 is 94% and 84%, and 94% and 89% for ricin E, respectively [67]. Starting from a cultivar expressing all three molecules, the presence of ricin E concomitant to RCA120 cannot be clearly differentiated and quantified from ricin D by LC-MS/MS on the protein level. Therefore, a more straight-forward strategy for the production of a ricin CRM is to start with the cultivar *R. communis zansibarienis* which has been shown to produce ricin D only [68]. Here, ricin D and RCA120 can be separated in sufficient amount and purity by preparative chromatography.

Due to gene synthesis, recombinant expression of a toxin offers maximal freedom with respect to selection of the variant. One criterium would be the epidemiological occurrence of the variant, but also biosecurity aspects need to be considered for selecting the toxin variant. As an example, BoNTs pathogenic to humans occur in more than 40 different variants called subtypes, which vary up to 36% on the amino acid level [69]. Subsequently, molecule characteristics like high specific toxicity to humans (to stress the sensitivity of methods to be validated), high water solubility, sufficient stability with respect to protein mass concentration as well as specific toxicity (in commonly usable formulations) and acceptable yield by the production process guide the selection process.

3) A third critical point to consider is the **molecular integrity** of the toxin CRM at the end of the production process. It has to be made sure that the production process (including purification/expression, filling, storage) does not compromise the identity and function of the analyte. To this end, the comparability of the toxin

CRM to its natural, authentic analog should be demonstrated by a panel of different methods (see below). Generally, the formulation of the toxin CRM, *e.g*., the buffer composition in case of a CRM solution, has to be carefully selected so that a comprehensive characterization of the CRM is technically feasible. It has to be considered that additives might stabilize the toxin CRM, but might interfere with certain measurement procedures (*e.g.,* addition of protein stabilizers or salt might interfere with MS-based methods, detergents impair analysis in cell-based methods); therefore, the use of additives should be minimized.

4) Finally, the whole production chain for toxin CRMs requires appropriate **safety and security measures** in place including a concept for safe storage and distribution. This goes beyond conventional safety and security measures since the large-scale production of toxin CRMs imposes additional challenges, namely upscaling issues. Generally, working with biological toxins requires physical security measures such as working in highly secured laboratories equipped with biological safety cabinets for delicate sample handling and having decontamination agents available in case of spill. In addition to these biosafety measures, biosecurity measures have to be implemented, such as facility security plans (*e.g.*, data and IT security, emergency response plans, procedures for receipt, transfer and shipment of select agents), personnel access control, personnel registration and security vetting, operational control and regular staff training. Additionally, risk assessments for all procedures need to be documented prior to the practical work. This includes information on adequate personal protective equipment, decontamination procedures and operational safety measures. There are useful references, which describe safety and health considerations for conducting work with biological toxins [70-72].

Characterization of Toxin CRMs

As mentioned above, availability of and accessibility to high-performance methods are key for the characterization of toxin CRMs. Apart from measurements for homogeneity, stability and characterization, this also accounts for the purity and identity assessment of the produced toxins. A variety of methods has to be applied to demonstrate identity and suitable purity of the preparations (*Figure **2***). These comprise but are not limited to liquid chromatography – (tandem) mass spectrometry (peptide fingerprinting and protein sequencing for identification; LC-MS of intact protein for exact mass determination), MALDI-TOF MS and other MS techniques to detect protein or peptide impurities, immunochemical methods such as ELISA and Western Blot as well as SDS-PAGE and/or capillary gel electrophoresis for a purity profile. In addition, for

glycosylated protein toxins a set of methods is required dedicated to comprehensive glycosylation analysis (identification of glycans and glycoforms). Typically, LC-MS methods are applied to identify the glycans, their structures and microheterogeneity, and to identify which N-glycosylation sites are occupied.

The mass concentration of the protein toxin in solution will best be quantified using protein impurity corrected amino acid analysis. It involves quantification of constituent amino acids following complete hydrolysis of the material and correction for amino acids originating from inherent structurally-related protein impurities. Individual amino acids are separated by liquid chromatography or gas chromatography (GC) and quantified, typically using isotope dilution mass spectrometry [62, 73-75]. The protein mass concentration is calculated taking into account the amino acid sequence of the protein. This approach requires a highly purified protein preparation; therefore the above-mentioned analyses are performed first to confirm a high level of purity. The speed and completeness of acidic hydrolysis of the protein to amino acids, both indispensable requirements for obtaining correct results, is sequence-depending, thus hydrolysis might need optimization [76].

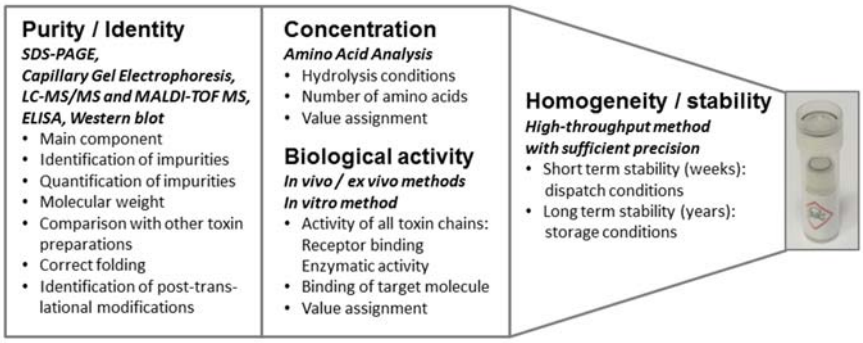

Figure 2. Characterization of toxin CRM requires a comprehensive and complementary panel of different methods.

Finally, different functional assays are required to assess the toxicity and/or biological activity of the toxin preparation. This is challenging since it is not obvious to link the precise protein amount to its biological activity – the outcome of activity determination heavily depends on the method used. For example, for ricin the measurement of cytotoxicity *in vitro* displays the activity of the A and the B chain (cell binding plus enzymatic activity), while adenine release assay indicates enzymatic activity only [19-20]. Likewise, for BoNTs it has been shown that the potency of different serotypes relative to each other is different by its quantitative factor when either *in vivo* (mouse bioassay) or *ex vivo* (mouse phrenic

nerve assay) methods are used [61]. This can be explained by the fact that the mouse bioassay describes the pharmacodynamics plus the pharmacokinetics of BoNT including absorption, distribution, metabolism and elimination from circulation of the individual BoNTs, while factors such as distribution, metabolism or elimination are not displayed in the mouse phrenic nerve assay. This discrepancy was extended by comparing the biological activity of the six native BoNT/A-F in *in vitro* cell-based assays (neurotransmitter release vs. substrate [SNARE protein] cleavage), *ex vivo* MPN assay and *in vivo* sublethal digital abduction score assay [77]. With respect to value assignment in a toxin CRM project it is therefore imperative to describe the functional method used in great detail so that it can be reproduced by the CRM customer.

Special Consideration of the Use of Toxin CRMs in Mass Spectrometry

From the different methods in use for detection of biological toxins, MS-based methods clearly display the highest specificity and allow for unambiguous identification of the analyte [23-24, 78-79]. Here, the availability of toxin CRMs is highly useful to develop the technology forward in terms of toxin identification, quantification and sample preparation.

Toxin identification is typically based on peptide mass fingerprinting of digested protein matched against the theoretical peptide masses derived *in silico,* nowadays often realized in MS/MS-based approaches. The availability of pure CRMs makes it possible to generate high-quality peptide spectral libraries for forensic identification of closely related protein toxins. Based on an *in silico* digest, it is important to determine which of the theoretically possible prototypic peptides can be measured experimentally with sufficient intensity and/or sensitivity depending on the instrumentation used. This information is a prerequisite to select diagnostic peptides for an overall workflow to be applied in the course of a forensic investigation [80-82]. Here, a regular verification of the selected diagnostic peptides against peptides derived from newly identified proteins is recommended to maintain specificity and hence unambiguous identification of the toxin [80].

Many state-of-the-art MS-based approaches are quite sensitive (low limits of detection and quantification). This can trigger matrix effect issues if a highly pure toxin is present at very low mass concentration in a buffer/matrix (unfavorable mass ratio of matrix to analyte). Highly pure toxin CRMs are important for the improvement of analytical procedures with respect to developing sample preparation methods, assessing recovery and in documenting the overall

efficiency and reproducibility of the workflow. Toxin CRMs will furthermore support the determination of the enzymatic digestion efficiency in order to obtain high sequence coverage.

Conclusions and Outlook

CRMs, thoroughly validated methods and PT studies are cornerstones of applied quality assurance**.** They are important QC tools for laboratories to validate and safeguard analytical methods. One of the main deliverables in the EuroBioTox project is the development and production of CRMs for plant and bacterial protein toxins. As in other fields, ideally pure substances for calibration (calibrants, either solutions or solid materials) as well as matrix RMs containing the analyte in a defined amount in a complex matrix representing a typical sample material (*e.g.,* toxin spiked into serum, food matrix, water) for method validation would be available. Producers of high-quality CRMs face several challenges regarding the purification or production process and the choice of the toxin variant to be produced. Safeguarding the molecular integrityof the analyte during the production process as well as high purity and biological activity of the CRM are key elements of the process. Above all, appropriate safety and security measures have to be in place to deal with the task.

The provision of such materials shall improve the preparedness and competence of laboratories to reliably analyse samples for biological toxins. Application fields are not limited to the food safety and public health sectors, but are equally important in security, military, and verification sectors. Overall, harmonized and standardized laboratory detection will enhance preparedness and response planning and will help to maintain a high level of vigilance, thus increasing resilience of the civil society in the capacity to prepare and respond to an incident involving biological toxins.

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Abbreviations

BoNT	Botulinum neurotoxin
CCQM	Consultative Committee for Amount of Substance: Metrology in Chemisty and Biology
CRM	Certified reference material
ELISA	Enzyme-linked immunosorbent assay
EQuATox	EU-project, acronym: Establishment of Quality Assurances for the Detection of Biological Toxins of Potential Bioterrorism Risk
EU	European Union
EuroBioTox	EU-project, acronym: European Programme for the Establishment of Validated Procedures for the Detection and Identification of Biological Toxins
GC	Gas chromatography
HC	Heavy chain of BoNT
LC	Light chain of BoNT
LC-ESI MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization—Time of Flight
MHC	Major histocompatibility complex
MS	Mass spectrometry
NAP	Neurotoxin-associated proteins
NMIs	National Metrology Institutes
PT	Proficiency test
QC	quality-control
RM	Reference material
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Staphylococcal enterotoxin
TCR	T cell receptor

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