Three Rs Approaches in Marine Biotoxin Testing

The Report and Recommendations of a joint ECVAM/DG SANCO Workshop (ECVAM Workshop 55)1, 2, 3

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Preface

This is the report of the 55th of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). The main objective of ECVAM, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences, and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures that would enable it to become well informed about the status of non-animal test development and validation, and of opportunities for the possible incorporation of alternative methods into regulatory procedures. It was decided that this would be achieved through a programme of ECVAM workshops, each addressing a specific topic, and at which selected groups of independent international experts would review the current status of various types of *in vitro* tests and their potential uses, and make recommendations about the best ways forward (1).

The workshop on Three Rs Approaches in Marine Biotoxin Testing was held at ECVAM on 24–26 January 2005, with participants from academia, national and international reference laboratories, competent authorities and the European Commission (EC). This ECVAM/DG SANCO Workshop was organised: a) to discuss the current state-of-the-art with regard to test methods and strategies; b) to consider immediate opportunities to reduce and refine animal use; and c) to examine the possibilities of validation and acceptance of non-animal methods as monitoring tools and reference methods in marine biotoxin testing, reflecting the technical progress that had been made. The outcome of the discussions and the recommendations agreed by the workshop participants are summarised in this report, which is primarily focused on the situation

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1ECVAM = European Centre for the Validation of Alternative Methods, DG SANCO = Directorate General for Health and Consumer Protection.
2This document represents the agreed report of the participants as individual scientists.
3The use of terms such as “validation”, “validation process” and “validation criteria” in this report is in accordance with the practices of the Association of Official Analytical Chemists (AOAC International), and differs in various ways from the use of similar terminology in relation to the testing of chemicals and chemical products, with which readers of ECVAM workshop reports are likely to be more familiar.
in the European Union (EU) and its Member States (MSs).

**Introduction**

Marine biotoxins, also known as shellfish toxins, are mainly produced by algae or phytoplankton. Based on their chemical structure, the toxins are classified into eight groups, namely, the Azaspiracid (AZA), Brevetoxin, Cyclic Imines, Domoic Acid (DA), Okadaic Acid (OA), Pectenotoxin (PTX), Saxitoxin (STX) and Yessotoxin (YTX) groups, as agreed at an FAO/WHO/IOC workshop held in 2004 (2). STX and its derivate belong to the Paralytic Shellfish Poison (PSP) toxin group, and DA to the Amnesic Shellfish Poison (ASP) toxin group. The Diarrhoieic Shellfish Poison (DSP) toxin group comprises OA and Dinophysis Toxins (DTX); together with lipophilic toxins from the AZA, PTX and YTX toxin groups, they can be accumulated in the tissues of filter-feeding molluscan shellfish, such as mussels, oysters, cockles, clams and scallops, and pose a health risk to humans if contaminated shellfish are consumed. Marine biotoxin-related illness can range from headaches, vomiting and diarrhoea to neurological problems, and, in extreme cases, can lead to death (3).

To protect public health, monitoring programmes for marine biotoxins have been established in many countries, which often stipulate the use of animal models (for example, the mouse bioassay [MBA] and the rat bioassay [RBA]), for detecting the presence of marine biotoxins in shellfish tissues. In the EU, only Germany has abandoned the *in vivo* tests and relies on biochemical and analytical methods, for which it had been criticised by the EC (4).

Various stakeholders (regulators, animal welfare organisations, scientific organisations) have expressed their concerns about the current legislation in Europe, not only with regard to the use of large numbers of animals, involving procedures which cause significant pain and suffering even though non-animal based methods are available, but also since the scientific community believes that the animal test may not be suitable for all classes of toxins (2, 5), and that the state-of-the-art scientific methodology for the detection and determination of marine biotoxins is not fully reflected in current practices.

**The Regulatory Framework in the EU**


**Council Directives on the monitoring of marine biotoxins and its supervision**

According to Chapter V in the Annex to the *Directive 91/492/EEC*, biological methods should be used to determine the total content of marine biotoxins belonging to the PSP and DSP toxin groups in live bivalve molluscs intended for immediate human consumption or further processing, whereas ASP levels are measured with analytical methods, i.e. by High Performance Liquid Chromatography (HPLC). In the case of the PSP group, any other method approved by the Standing Veterinary Committee (recently replaced by the Standing Committee on the Food Chain and Animal Health [SCFCAH]), as detailed in Article 12 of the Directive may be used; however, if the results are challenged, the biological test method must be the reference method (for more details see *Animal tests*).

*Commission Decision 2002/225/EC* includes more details concerning the implementation of *Council Directive 91/492/EEC*. It defines the maximum levels for the toxins previously comprising the DSP (OA and DTX), YTX, PTX and AZA groups, and the detection methods (Annex to the Decision). The term *biological methods* covers several *in vivo* assays, including the MBA and the RBA. The Decision permits the use of *alternative detection methods*, and names various analytical methods, functional assays and immunoassays, provided that they have been validated in compliance with an internationally accepted protocol. However, Article 5 of *Decision 2002/225/EC* explicitly states that, in case of discrepancies between the results obtained with different methods, the DSP MBA should be considered as the reference method for the biotoxins specified in the Decision.

It should also be borne in mind that the Competent Authorities (CAs) of the MSs are responsible for the methods to be used for the periodic monitoring of live bivalve molluscs relaying and production areas (*Directive 91/492/EEC*, Annex, Chapter VI), and thus have the opportunity to use non-animal methods for some applications.

It is noteworthy that no animal tests are required for the monitoring of ASP, and that ASP levels are determined by using HPLC methods, as laid down in *Council Directive 97/61/EC* (11).

In 2004, the Community Legislature passed the so-called *EU Hygiene Package* of regulations (12), bringing together and replacing the existing hygiene regulations for the food sector currently...
contained in numerous individual guidelines. Regulation (EC) 853/2004, Annex III Section VII Chapter V No. 2 (13), lays down the maximum levels for ASP, PSP and DSP toxins. Article 11, referring to Article 12 of the same regulation, entails authorisation to specify approved analysis methods for marine biotoxins. Regulation (EC) 854/2004, Annex II Chapter II (14), gives the monitoring authorities in the EU MS the mandate to examine live molluscs for the presence of marine biotoxins. The EU Hygiene Package came into effect on 1 January 2006.

The Community Reference Laboratory and National Reference Laboratories

Council Decision 93/383/EEC (15) stipulates, inter alia, that a National Reference Laboratory (NRL) shall be established in every MS, to ensure the application of effective supervision procedures for the testing for marine biotoxins, and to coordinate the conduct of the necessary analyses within the national laboratories.

In order to guarantee uniformity in the test procedures for marine biotoxins within the Community, a Community Reference Laboratory (CRL) has also been established. Furthermore, Decision 93/383/EEC (15) stipulates that, apart from co-ordinating the application of test procedures, the exploration of new analytical methods, and informing the NRLs about any progress made, will also be part of its responsibilities. The laboratory of the Ministerio de Sanidad y Consumo in Spain was designated as the CRL in 1993. Since 2005, the CRL has been part of the Spanish Food Safety Agency of the Spanish Ministry of Health. The duties of the CRL are summarised in the legislation as follows:

— supplying information on analytical methods and comparative testing to the NRLs;

— coordinating the application by the NRLs of the above methods by organising comparative testing;

— coordinating the development of new analytical methods and informing the NRLs of progress made;

— organising training and advanced courses for the staff of the NRLs;

— collaborating with the laboratories responsible for analysing marine biotoxins in other countries outside the EU;

— providing scientific and technical assistance to the EC, especially in cases where the results of analyses are contested among the MSs; and

— helping the NRLs to implement an appropriate system of quality assurance, based on the principles of Good Laboratory Practice (GLP) and the EN 45 000 criteria; while

In addition, Article 5 of Decision 93/383/EEC (15) states that the CRL for marine biotoxins shall satisfy the following operating conditions:

— that staff are qualified and have sufficient knowledge of the techniques applied in the analysis of marine biotoxins;

— that the equipment and substances necessary for carrying out the tasks laid down in Article 4 of Decision 93/383/EEC (15) are available;

— that an appropriate administrative structure is in place;

— that the confidential nature of certain subjects, results and reports is observed by the staff;

— that the internationally accepted principles of GLP are followed; and

— that an up-to-date list of the reference substances held by the Community Bureau of References (now the Institute for Reference Materials and Measurements [IRMM], EC DG Joint Research Centre, Geel, Belgium) are available, along with an up-to-date list of the manufacturers and suppliers of these substances.

The CRL has recently undergone a number of changes, including the incorporation of more staff, and better facilities and equipment, and is working on designing a mechanism for total replacement of the bioassay for each group of toxins. Therefore, in the near future, the MBA should have a replacement, or a procedure should be in place which would allow its replacement as soon as is technically possible.

The Food and Veterinary Office (FVO)

The Food and Veterinary Office (FVO) of DG SANCO is responsible for supervision of the testing and monitoring programmes concerning fishery products and bivalve molluscs, to ensure protection of consumer health in Europe. The FVO regularly carries out inspections in the EU MSs in cooperation with the national coordinators. (16)

The reports of inspections conducted in 2002 indicate that, despite the provisions of Directive 2002/225/EC, a variety of test methods and strategies are in use across the EU. Mainly due to the difficulties inherent in animal testing, some of the MSs do not currently comply satisfactorily with the...
procedures stipulated in the EU marine biotoxin monitoring legislation (N.B. inspection reports are available at http://europa.eu.int/comm/food/fvo/ir_search_en.cfm [17]).

The Council Directive on the protection of laboratory animals

Council Directive 86/609/EEC (10) makes provision for laws, regulations and administrative provisions for the protection of animals used for experimental and other scientific purposes. This includes the use of live vertebrate animals as part of testing strategies and programmes to detect, identify and quantify marine biotoxins. Indeed, the scope of Article 3 of the Directive includes the use of animals for the safety testing of food, and the avoidance of ill health and disease. The EC has also signed and ratified the Council of Europe Convention ETS 123 (18).

Directive 86/609/EEC sets out the responsibilities that the MSs must discharge. As a result of this use of prescriptive language, the MSs have no discretion or flexibility, and most of the provisions of the Directive must be applied in all cases. It is clear that the MSs have to ensure that: the number of animals used for experimental and other scientific purposes is reduced to the justifiable minimum; that such animals are adequately cared for; and that no unnecessary or avoidable pain, suffering, distress or lasting harm are caused in the course of such animal use.

The MSs may not (Article 7, 2) permit the use of live animals in procedures that may cause pain, suffering, distress or lasting harm, “if another scientifically satisfactory method of obtaining the result sought and not entailing the use of live animals is reasonably and practicably available”. The EC has previously advised the MSs that the fact that an animal test is specified as part of a regulatory test programme does not absolve a MS of its responsibilities under Directive 86/609/EEC to ensure that all animal use meets this requirement. The EC has further advised that animal testing should not be permitted, once an alternative has been scientifically validated and an international protocol has been made available.

When animal use can be justified, Directive 86/609/EEC specifies a range of safeguards that MSs must put in place to avoid or minimise any animal suffering that may be caused. All justifiable animal use should be designed and performed to avoid unnecessary pain, suffering, distress and lasting harm (Article 8). The MSs must ensure (Article 19, 1) that user establishments undertake experiments as effectively as possible, with the objective of obtaining consistent results, whilst minimising the number of animals and any suffering caused. This latter requirement necessitates the use of minimum severity protocols, including appropriate observation schedules, and the use of the earliest humane endpoints that prevent further suffering, once it is clear that the scientific objective has been achieved, that the scientific objective cannot be achieved, or that the suffering is more than can be justified as part of the test procedure.

The EC and the MSs are also required (Article 23, 1) to encourage research into, and the development and validation of, alternative methods that do not require animals, use fewer animals, or further reduce the suffering that may be caused, whilst providing the same level of scientific information.

Description and Status of Methodology

For ethical and scientific reasons, intense efforts have been made during the past years to develop suitable approaches to refine and/or to reduce reliance on in vivo testing. A large variety of approaches have been described, a number of which appear promising supplements to or even replacements of the MBA.

Animal tests

Animal tests as currently prescribed in legislation

PSP toxin group

Directive 91/492/EEC specifies the MBA for the detection of toxins belonging to the PSP group (STX-group) as the reference method for end product testing. The total PSP toxin content in edible parts must not exceed 80µg/100g mollusc flesh, in accordance with the MBA — in association, if necessary, with a chemical method for detection of STX — or any other method recognised in accordance with the procedure laid down in Article 12 of the Directive.

The PSP MBA corresponds to the AOAC (Association of Official Analytical Chemists) Official method 959.08 (19) and has been in use for more than fifty years in many of the MSs. Its limit of detection is approximately 40µg STXeq/100g shellfish tissue, which is 50% of the current maximum residue limit (MRL), and it has a precision of 15–20% (20–23). The method may underestimate true levels, for example when levels of about 80µg/100g mollusc flesh are detected by the PSP MBA, the actual concentration present may range from 1.2 to 2.0µg/kg, due to salt effects (2).

The method states that, initially, one, but preferably two or three, mice are injected intraperitoneally (i.p.) with 1ml of an acid extract (pH 2–4) of shellfish. The death time is recorded, and if < 5 minutes, dilutions have to be made until a death time of 5–7 minutes is achieved. Then, three mice
are injected and the median death time is determined.

The toxic concentration is determined from Sommer's Table, in mouse units (MU), multiplied with the dilution factor and, if necessary, a weight correction, giving the result in MU/100g. Conversion from MU to µg STX equivalents (eq) is achieved by multiplying with a conversion factor (CF), which has to be established for each laboratory (and checked regularly).

It is recognised that the PSP MBA produces rapid and reasonably accurate results, but some reports indicate a significant interlaboratory variation (20, 23, 24). However, it is painful to the animals, stipulates death as an endpoint, and requires large numbers of mice. The number of mice can be reduced by omitting the initial dilution step(s), since a survival time < 5 minutes equals > 64µg STXeq/100g, which is close to the survival time corresponding to the MRL. Further drawbacks with the PSP MBA are that toxicity could be underestimated due to high salt concentrations, and false positives may appear, due to the natural accumulation of zinc, mainly in oysters (25). Other authors report that there is evidence of variability in results, due to mouse strain and sex differences (21, 26, 27).

**DSP toxin group and other lipophilic toxins**

Marine biotoxins of the DSP group (OA, DTX), AZA and, possibly, YTX and PTX pose a serious hazard to human health when present above certain limits in bivalve molluscs, echinoderms, tunicates or marine gastropods. Decision 2002/225/EC sets the following maximum levels:

- total of OA/DTX and PTX shall not exceed 160µg of O Aeq/kg;
- YTX and derivates shall not exceed 1mg YT Xeq/kg; and
- AZA shall not exceed 160µg of AZA-eq/kg.

A series of MBA procedures, differing according to the test sample (hepatopancreas [HP] or whole body [WB]) and to the solvents used for extraction and purification steps, can be used for the detection of the DSP toxin group and other lipophilic toxins (covered by the term DSP MBA in the following discussion). The selectivity of the assay depends on the choice of the solvents used and whether WB or HP is used as the sample. This should be taken into account when making a decision on the method to be used, in order to cover the full range of biotoxins. For example, a single DSP MBA involving acetone extraction can be used to detect OA/DTX, PTX and YTX, whereas a single DSP MBA with acetone extraction followed by L/L partitioning with diethylether, can be used to detect OA/DTX, PTX and AZAs, but not YTX. AZA detection requires the use of a WB sample.

In general, three mice should be used for each test. The death of two out of three mice within 24 hours of the i.p. injection of 5g HP-eq or 25g WB-eq should be considered as a positive result.

An animal test with three rats (rat bioassay [RBA]) can be used to detect OA/DTXs and AZAs (8).

In recent years, the pros and cons concerning animal-based tests have been discussed, not only with regard to animal welfare concerns, but also regarding their appropriateness for detecting toxins at levels which threaten human health. A potential advantage of the DSP MBA is that it might detect unknown lipophilic toxins due to unusual symptoms, and that it can be cheaper compared to analytical methods based on advanced instruments. However, it causes substantial animal suffering, and the testing requires many mice (three mice/sample, and an additional three mice, if the presence of YTX is suspected; according to Protocol 2 of Yasumoto in 2001 [T. Yasumoto, personal communication]). Due to the lack of specificity of the DSP MBA, it is not possible to distinguish which of the lipophilic toxins contribute to the toxic effects observed, and both false negatives and false positives are known to occur. It is therefore not fit for its stated purpose, namely, to detect OA, PTX, YTX and AZA and to ensure that the maximum levels are not exceeded. The DSP MBA was initially developed as a research tool in toxicology to detect OA and DTX toxins, and not necessarily as a method for routine monitoring to protect against DSP caused by these two toxin classes. The design of the MBA reflects implicitly the correlation between the oral toxicity of OA and DTXs in humans and the i.p. toxicity of OA and DTXs in mice. Lipophilic toxins other than OA and DTX exert toxicity via different mechanisms of action, so the relationship between i.p. toxicity in mice and oral toxicity in humans for these toxins differs from that for OA and DTX.

In addition, cyclic imines (gymnodimine, spirulines and others), which are currently not regulated in the EU, are detected in the DSP MBA. The data available for some of the cyclic imines show that they are more toxic when i.p. injected than when administered orally. Consequently, the cyclic imines may contribute to the overall outcome of the DSP MBA, which is intended to quantify only lipophilic toxins regulated by the EU, although it is highly improbable that they present a health risk at this low level (according to the FAO/IOC/WHO draft monograph [2]). Furthermore, the effects of combinations of toxins are unknown.

If the recommendations from the FAO/IOC/WHO Expert Consultation meeting (2) are implemented, the MRLs for different toxin groups will be reduced, and the DSP MBA would no longer be relevant for routine purposes and could only be used for screening for new toxins.
The DSP MBA is not harmonised in the EU, and several main protocols are available, mostly based on methods developed by Yasumoto for DSP toxins (28, 29). Even if laboratories use the “same” main protocol, many factors differ between laboratories, including strain and sex of the mice used. Also, none of the DSP MBA protocols have undergone formal validation. The EU CRL and NRLs undertook some efforts to harmonise the in vivo test for lipophilic marine biotoxins, and, in 2004, the Working Group established for this purpose, suggested a Standard Operation Procedure (SOP) for the detection of OA/DTX, PTX, YTX and AZA, based mainly on the work of Yasumoto et al. (29) and Yasumoto protocol 2 (T. Yasumoto, personal communication).

It would be an advantage to have an EU-wide harmonised protocol for the detection of DSP toxins and other lipophilic toxins based on either WB or HP; however, this would not overcome the drawbacks listed above. The initial draft protocol even suggested that only mice with 20 ± 1g body weight should be used, thus not allowing a weight-adjusted injection. Weight-adjusted injections would save a large number of mice in some laboratories, and have been used in the Norwegian reference laboratory for several years, based on studies which revealed no problems in using mice weighing 16–23g (30). A widening of the weight range of mice to be used in the assay has been recommended by the 2005 Working Group of the EU NRLs, as part of a further harmonised protocol.

Conclusions and recommendations

The RBA is not commonly used. It is less painful for the animals, but it can only be used for the detection of toxins belonging to the OA and AZA groups. It is a semi-quantitative test and, like the DSP MBA, has never been formally validated. Its relevance could increase, if MRLs for YTX and PTX were to be changed according to the recommendations of the FAO/IOC/WHO Expert Consultation meeting (2).

Up to now, the DSP MBA as prescribed in the legislation, has not been harmonised or validated across Europe or internationally. One of the main flaws in the currently applied protocols remains the false positives resulting from the high sensitivity of the DSP MBA toward YTXs and cyclic imines, which are either regulated at higher levels than the assay detects or not regulated at all. The other main drawback is the tendency for false negatives resulting from the poor sensitivity of the assay toward some toxins of the OA group, especially esters of OA and DTXs (31), but potentially also for the AZA group (32).

Due to the non-specific nature of the DSP MBA, the inability to implement levels currently regulated, the fact that the test cannot be validated, and the continued prolonged exposure of large numbers of mice to substantial pain and suffering while non-animal based methods are being validated, it is recommended that the DSP MBA is replaced as quickly as possible, and that, if necessary, a stepwise approach is undertaken for different circumstances, e.g. screening of production areas.

Refinement and reduction of the mouse bioassays

As mentioned above, a large number of animals are used for marine biotoxin testing, and the stipulated in vivo tests cause substantial suffering and pain to the animals. In the following discussion, two approaches are described, which could refine the PSP MBA and reduce the number of mice used for DSP testing.

Refinement of the PSP MBA

A terminally anaesthetised mouse model has been developed by Dennison et al. (33) for PSP testing. Mice are anaesthetised prior to the i.p. injection of the test sample, and remain anaesthetised until the test is completed. Other than minor discomfort at the time the anaesthetic is administered, no animal suffering is involved, and anaesthesia eliminates the otherwise substantial welfare costs associated with the standard PSP MBA. The method seems to produce sufficiently robust data to underpin the required risk assessment. There is evidence from work with STX that the use of anaesthesia may increase the sensitivity of the PSP MBA and therefore make it more reliable than the conscious model (34, 35).

It is recommended that the CRL/NRL should evaluate how this refinement method could be incorporated into the current PSP MBA protocol, unless the short-term replacement of the in vivo test is possible.

Reduction of the DSP MBA and the PSP MBA

The British National Reference Laboratory for Marine Biotoxins (36) is supportive of a statistically sound approach for the evaluation of test data, which facilitates the use of fewer animals in the DSP MBA than stipulated (c.f. Council Directive 86/609/EEC, Articles 7 and 19 [10]).

For a positive result, the standard DSP MBA requires the death of two out of three mice within 24 hours after i.p. injection of a shellfish extract. An analysis of samples tested by one of the UK monitoring laboratories indicated that the use of two mice gave an unequivocal result (both mice alive or both mice dead at the end of the test) for over 99% of the samples tested. Based on the percentage of positive samples, the use of a third mouse was only justified in 1 in 1000 samples (where one mouse was alive and one dead at the end of the test period). In the event of an equivocal result, a third mouse can be used. However, it is not necessary to inject the
additional animal, if the criterion applied is that a positive result is recorded where one out of two mice dies rather than two out of three, since this is always safer, so the sensitivity required by the EC directive is exceeded.

In recent years, in the light of the above findings, the British monitoring laboratories have reduced the number of animals per test for DSP toxins to two, thus reducing the numbers of animals used in the UK by approximately 33%.

In addition, Asp et al. (37) have described the reduction of the numbers of mice required in the PSP MBA by omitting the initial dilution step(s). Although, the PSP MBA was performed according to the AOAC method (19), the authors emphasised that it is unnecessary to perform the initial toxin titration in order to dilute highly toxic samples, until the survival times of the mice are between 5 and 7 minutes. The reason for this is that the MBA is used to safeguard consumers of shellfish against PSP, and even though the precision is reduced when injecting undiluted samples with high toxin concentrations, the precise amount of toxin is of less importance, as long as samples are rejected when the tolerance level is exceeded.

**Recommendation**

It is recommended that the CRL/NRLs should evaluate how reduction of the DSP MBA and PSP MBA could be incorporated into the current protocols; however, this should not be permitted to delay the validation of replacement methods.

N.B. At the NRLs meeting in November 2005, it was decided to amend the requirements for DSP MBA, so that two mice are injected initially. A third mouse is only needed, if one mouse survives and the other dies. This amendment should come into force in 2006 (Luis Botana, personal communication).

**Non-animal methods**

As outlined under **Regulatory Framework**, above, **Commission Decision 2002/225/EC** (8) foresees the use of non-animal based methods for DSP detection and refers in its Annex on **Detection Methods** to alternative detection methods. It states that a series of methods (HPLC, liquid chromatography and mass spectrometry [LC-MS], immunoassays and functional assays) can be used as alternative or complementary methods. Such methods should undergo validation according to internationally agreed criteria. They should detect, either alone or in combination with other methods, a number of toxins and their analogues:

- OA and DTXs (a hydrolysis step may be required in order to detect DTX3);
- PTXs: PTX1 and PTX2;
- YTXs: YTX, 45-OH-YTX, homo-YTX, and 45-OH-homo-YTX;
- AZAs: AZA1, AZA2 and AZA3.

They should be at least as effective as the biological method and provide an equal level of public health protection. Newly detected analogues should be included in the analysis. The total toxicity will be calculated by using relative toxicity factors based on the toxicity data available. However, the Decision also states that, when the results of the analyses performed demonstrate discrepancies, the biological method should be considered as the reference method.

With regard to PSP, alternative methods should be approved by the SCFCAH (see **Regulatory Framework**), and here also, the *in vivo* method is named as the reference method when the results obtained with alternative methods are challenged.

The following is a summary of the currently available non-animal based methods for detecting and quantifying the eight marine biotoxin groups and their suitability for monitoring and end product testing of molluscs, with a particular focus on their potential to reduce the use of animal testing. It is in line with the review of the FAO/WHO/IOC Expert Consultation meeting held in Oslo in 2004, to provide guidance to the Codex Alimentarius Commission on methods of analysis for marine biotoxins in shellfish (2).

**Functional methods**

Biologically active substances, including marine biotoxins, trigger specific responses by interacting with a cellular component that selectively recognises their structures and behaves as a receptor in sensitive systems, thereby transforming the chemical information of the incoming ligands into defined cellular effects (38).

Functional methods exploit the high selectivity of receptor systems in the recognition of structural properties of their ligands. Furthermore, functional methods rely on the capacity of receptors to integrate the relative potencies of the biologically active analogues of the chemical class present in a single sample, and to trigger an overall response in the sensitive system. Therefore, with functional methods, the analytical challenge posed by the extreme complexity of toxin profiles that may characterise natural samples, is handled by the biological system, whose responses are specific for any toxin group. Under these conditions, the response of the biological system is a hyperbolic function of toxin concentration, and the overall levels of biologically active analogues in unknown samples is then obtained by the quantitative determination of responses, yielding estimates of concentrations in “equivalents” of the reference toxin.
A number of functional methods exist for the detection of several marine biotoxin groups (reviewed in 38, 39). Table 1 summarises key features of major procedures available for the STX, OA and YXT groups. The methods make use of both intact cells and cell-free systems, and are mostly based on recognised molecular targets of the toxins.

**Saxitoxin (STX) group**
The inhibitory effect of STX on Na⁺ channels is targeted in measurements of either the cell survival after ouabain/veratridine treatment (40), or the change of membrane potential in veratridine-treated cells (41, 42). The involvement of a recognisable mechanism of toxin action in STX methods is matched by limits of detection of the procedures for STX group, spanning concentrations within one order of magnitude (Table 1). The detection step in the procedures involves direct colorimetric or fluorimetric analysis, and measurements of many samples can be performed in multi-well plates. Functional fluorescent methods based on the use of membrane potential dyes have also been developed for the detection of STX (41, 43). Furthermore, other procedures for the neuroblastoma assay have been developed for the detection of brevetoxins (44–47).

**Okadaic Acid (OA) group**
The OA group is quantified by its inhibitory effect on serine-threonine phosphoprotein phosphatase 2A (PP2A; 48–50). The limit of detection of the procedures for the OA group also covers concentrations within one order of magnitude (Table 1). The detection step in the functional methods for the OA group encompasses direct colorimetric or fluorimetric analysis. These analyses permit measurements of many samples in multi-well plates. When this format is used in rapid (1 hour) methods, the procedure is appropriate for screening a high number of samples. The PP2A-based functional methods for the detection of OA and its analogues are being commercialised, and are likely to be validated in the near future.

**Yessotoxin (YTX) group**
In the case of the YTX group, one method is based on its ability to enhance phosphodiesterases acting on cyclic AMP (51), whereas the proximal molecular target (receptor) of YTX in the method based on the measurement of E-cadherin fragmentation in epithelial cells is still unknown (52, 53). Biochemical methods for phosphodiesterase (PDE) enhancement-based assays are also available (see the following section on Biochemical/immunological methods). The limit of detection of functional or biochemical methods for YTXs differs by three orders of magnitude (51, 52, 54), and this might be explained by the multiple molecular targets that have been proposed as playing some role into the actions of the toxin (53, 55–57). The detection step in the procedures involves the immunoblotting of cell extracts (52) or fluorimetric analysis (51). Fluorimetric analysis allows the rapid (1 hour) measurement of many samples in multi-well plates. Therefore, the procedure is appropriate for screening a large number of samples. Furthermore, an interlaboratory study to characterise the performance of the functional method to detect YTX based on E-cadherin fragmentation in cultured cells should be part of a project sponsored within the EC’s 6th Framework Programme FP6; 58.

**Functional methods and toxic equivalence factors**

One obstacle hampering the full characterisation of method accuracy is probably related to the fact that toxic equivalence factors (TEF) to be used in calculations have not been obtained for many toxin analogues. TEFs represent the potency of an analogue as compared to that of a reference compound (see below). Therefore, an accurate characterisation of method performance demands that TEFs are obtained for major toxin analogues for each toxin group. Due to the lack of TEFs, and thus the lack of comparability with other methods, these methods are still classified as having a tentative status in the context of Codex Alimentarius.

Literature data on the functional methods listed in Table 1 indicate a fairly good within-laboratory performance. While some of them have been subjected to interlaboratory validation exercises, the results of those studies have not yet been published. It can be concluded that many valuable alternatives exist for different classes of toxins, so that every procedure has a preferred field of application. However, the use of functional methods in marine biotoxin detection and measurement requires the formal validation of individual procedures according to internationally agreed criteria. Due to the particular features of functional methods, a rational scheme for the validation of any such method could involve three steps, including:

1. evidence that the method provides a satisfactory estimate of the analyte, when a single toxin is present in the material;

2. evidence of good reproducibility for materials containing a mixture of analogues; in these cases, the overall contents of toxins in individual materials should be assessed by a reference institution using the same functional method;

3. evidence that the estimates of analytes assessed in a sample by a confirmatory method can be converted into those obtained by the functional method through the mathematical treatment of data.

In our opinion, the validation of functional methods will be facilitated by further efforts in three
<table>
<thead>
<tr>
<th>Toxin group</th>
<th>Neuro-2a neuroblastoma cell line</th>
<th>BE(2)-M17 neuroblastoma cell line</th>
<th>Cell-free</th>
<th>Okadaic acid group</th>
<th>Cell-free</th>
<th>Cell-free</th>
<th>Cell-free</th>
<th>Yessotoxins</th>
<th>Cell-free</th>
</tr>
</thead>
<tbody>
<tr>
<td>System</td>
<td>CSVOT</td>
<td>MPVTC</td>
<td>MPVTC inhibition</td>
<td>PP2A inhibition</td>
<td>PP2A inhibition</td>
<td>PP2A fragmentation</td>
<td>E-cadherin enhancement</td>
<td>PDE</td>
<td></td>
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<tr>
<td>Principle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theoretical limit of detection</td>
<td>0.5ng/ml</td>
<td>1.0ng/ml</td>
<td>5.0ng/ml</td>
<td>0.2nM</td>
<td>0.2nM</td>
<td>0.1nM</td>
<td>0.3nM</td>
<td>0.5μM</td>
<td></td>
</tr>
<tr>
<td>Type of detection</td>
<td>Colorimetric</td>
<td>Fluorimetric</td>
<td>Fluorimetric</td>
<td>Colorimetric</td>
<td>Colorimetric</td>
<td>Colorimetric</td>
<td>Fluorimetric</td>
<td>Fluorimetric</td>
<td></td>
</tr>
<tr>
<td>Method format</td>
<td>96-well plate</td>
<td>Single sample</td>
<td>Single sample</td>
<td>Single sample</td>
<td>96-well plate</td>
<td>96-well plate</td>
<td>Single sample</td>
<td>96-well plate</td>
<td></td>
</tr>
<tr>
<td>Method duration</td>
<td>24 hours</td>
<td>1 hour</td>
<td>10 minutes</td>
<td>1 hour</td>
<td>1 hour</td>
<td>1 hour</td>
<td>48 hours</td>
<td>~1 hour</td>
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<tr>
<td>Throughput</td>
<td>High</td>
<td>Medium</td>
<td>Medium</td>
<td>Medium</td>
<td>High</td>
<td>High</td>
<td>Low</td>
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<td></td>
</tr>
<tr>
<td>Detection in spiked samples?</td>
<td>Yes (1)</td>
<td>NR</td>
<td>NR</td>
<td>Yes</td>
<td>Yes (2)</td>
<td>Yes (1)</td>
<td>Yes (3)</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>Detection in naturally contaminated samples?</td>
<td>Yes (12)</td>
<td>Yes (3)</td>
<td>Yes (n)</td>
<td>NR</td>
<td>Yes (30)</td>
<td>Yes (16)</td>
<td>Yes (21)</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>Limit of quantification</td>
<td>2.0μg/100g ST</td>
<td>0.2μg/100g ST</td>
<td>20.0μg/100g ST</td>
<td>100.0ng/g DG</td>
<td>1.0ngOA/g DG</td>
<td>12.8ng/g DG</td>
<td>100.0ng/g DG</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>Correlation with chemical analysis?</td>
<td>Yes</td>
<td>Yes</td>
<td>NR</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>International validation?</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
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<td>40</td>
<td>41</td>
<td>42</td>
<td>48</td>
<td>49</td>
<td>50</td>
<td>52</td>
<td>51</td>
<td></td>
</tr>
</tbody>
</table>

*a*the number of separate samples included in the study are shown in brackets; CSVOT = cell survival after ouabain/veratridine pretreatment; MPVTC = membrane potential in veratridine-treated cells; PP2A = phosphoprotein phosphatase 2A; PDE = phosphodiesterase; ST = shellfish tissue; DG = digestive gland; NR = not reported; n = not indicated.
areas, related to their development and implementation:

1. the estimation of TEFs for major analogues in each toxin class, and the characterisation of their performance in individual procedures;

2. the preparation of reference materials for use in the comparison of methods and in interlaboratory validation studies;

3. the increased dissemination of methods and concepts belonging to classical biochemistry and cell biology, which represent the background knowledge of functional methods, and would support a more thorough integration of toxicology and analytical chemistry.

Conclusions concerning functional methods
A thorough evaluation of method performance by the use of field samples containing complex toxin profiles has not yet been completed. While predictable responses are obtained with purified standards, the analysis of shellfish extracts, either spiked by the addition of reference compounds or naturally contaminated by toxins, has posed problems due to interference and other matrix effects (51, 52), so that the theoretical limit of detection is not invariably matched by the limit of quantification of toxins in field samples tested by using the individual procedures. The correlation of measurements performed by functional methods with estimates of toxin contents by chemical analyses of field samples, has been reported for the methods listed in Table 1.

Biochemical/immunological methods
Biochemical or immunological methods for the detection of marine biotoxins are based on the specificity of the binding process of compounds to antibodies. Antibodies can be either raised in animals (for example, polyclonal antibodies in rabbits, sheep and mice) without causing significant pain, or in cell cultures (such as monoclonal antibodies in hybridomas). After the isolation of the antibody from the cell culture supernatant or the animal’s serum, the antibody can be used in a variety of tests, including enzyme-linked immunosorbent assays (ELISA), lateral flow immunoassays (LFIA), and biosensors based on surface plasma resonance (SPR).

The nature of methods based on antibodies, i.e. chemical recognition by a non-functional molecule, means that the result of the test does not represent a direct measure of the toxicity present in a sample. In cases where there is only one toxin present and the antibody has been designed for this compound, the measure of the concentration of the toxin in the sample is directly correlated to its toxicity; this is to some extent the case for DA. In all other cases, the results of antibody-based assays can only be considered to be estimates of the toxicity present in a sample. Due to this pitfall, immunological methods can only be considered as screening or qualitative confirmatory methods, but not as quantitative confirmatory methods. However, with regard to the replacement of the MBA, methods for screening purposes are particularly important since most of the tests are carried out for this purpose. Also, immunological methods are particularly well suited to screening, thanks to their specificity, typically leading to the ability to detect very low concentrations.

Azaspiracid (AZA) group
AZAs are a relatively recently discovered group of toxins and, to date, no methods based on the use of antibodies have been published. Several groups are working in this field, and since the total synthesis of Azaspiracid-1 has been achieved (59, 60), a breakthrough in the area of antibodies can be expected over the next few years. An SPR-antibody based assay is being developed in the FP6 project, DetecTox (61).

Brevetoxin group
This toxin group is currently not directly regulated in the EU, because it occurs only in the USA and the South West Pacific Ocean. An ELISA has been developed for this group (62), and is currently under evaluation for use for official control purposes in the USA (63). The most recent views of the AOAC task force suggest that the ELISA may currently be the best available option, as compared to the American Public Health Association MBA or LC-MS (64).

Cyclic Imines group
These compounds have been discovered as a result of their rapid effects when injected into mice. Again, this group of compounds is not currently regulated in the EU, mainly because it has not yet been demonstrated that any of its constituents are of relevance to public health. No immunological methods are available for this group of compounds.

Domoic Acid (DA) group
A number of immunological methods are available for the detection of DA. These methods include Lateral Flow Immuno-Chromatography (LFIC; Jellett Rapid Test for ASP [65]), an ELISA (66), and SPR biosensor techniques (67). The current EU regulation prescribes analysis by HPLC (e.g. 68), mainly because the animal methods were deemed not to be sufficiently sensitive, so no animals are currently being used for the official control of these toxins. The ELISA is the method which is most advanced in terms of validation status through col-
laborative trials, and validation according to the AOAC criteria is expected within the next few years (69).

**Ohadaic Acid (OA) group**

The DSP MBA was originally developed for the OA group, and it is still being monitored in a number of EU MSs by using versions of this animal-based method. An ELISA is available for the OA group (70); however, the cross reactivity for the antibody was questioned for some of the analogues in the group, and the limit of detection is relatively poor (71). There has been no reported collaborative trial for this ELISA.

**Pectenotoxins (PTX) group**

PTXs are currently regulated in the EU, although no direct impact on public health has been demonstrated. This group could also be considered as interference in the DSP MBA, and therefore may not require regulation, once the animal test is replaced. The development of an antibody-based procedure for this group is under way (58, 61), but no validated method is yet available. An actin-based assay is being developed in the DetecTox project (61), which involves the use of SPR biosensor techniques.

**Saxitoxin (STX) group**

Two types of approaches have been pursued in the biochemical area for the STX group, namely, antibody-based and receptor binding methods. The antibody-based methods have been developed both as an ELISA and as a kit-format using LFIC, which has been trialled in the field extensively and which has also undergone some collaborative trials, the outcome of which awaits AOAC approval. LFIC kits have been developed, such as the Rapid Test for PSP (formerly, MIST Alert™ [40, 72]), with a detection limit of 40 µg/100g in less than 20 minutes. Field trial results indicate that the Rapid Test for PSP could also be used by shellfish farmers as a shellfish management tool, and by processors in end-product testing (73). Food Standards Agency Scotland (FSAS) performed the Rapid Test for PSP (74, 75) in parallel with the PSP MBS, and has proceeded to implement as of spring 2004. The lowest limit of detection obtained by an ELISA test was 0.2 ng/g of shellfish tissue (76). Based on this principle, a test kit is commercially available (the RIDASCREEN® Saxitoxin assay [77]) and has been evaluated in-house. The receptor-binding assay, initially described by Viytes et al. (78) and further developed by Doucette et al. (79), is now available in a radioactively-labelled isotope format (80), which shows very good precision and robustness, as well as an excellent comparability with the animal test. The receptor-binding assay for this particular toxin group can also be considered to be a biochemical method, since it is based on the binding to the molecular target responsible for the biological reaction (mode of action known). Following the successful completion of informal intercalibration exercises, a formal collaborative trial is planned for the near future (81). A SPR-biosensor-based antibody method is currently being validated in the FP6 project, BioCop (82).

**Yessotoxin (YTX) group**

This toxin group could also be considered to be mere interference in the DSP MBA, since no direct impact of these compounds on public health has been shown. Nevertheless, an ELISA was developed for this compound group, which has undergone an extensive single-laboratory evaluation and a limited collaborative (pre)validation study. It is expected that this method will be collaboratively validated over the next three years, within the FP6 project, BIOTOX (58).

As mentioned in the Functional Methods section, two other biochemical methods for the detection of YTX exist, based on the interaction of the toxin with phosphodiesterases acting on cyclic AMP. These methods involve different technological approaches, consisting of a resonant mirror biosensor (Pazos et al., 2004) and of fluorescence polarisation (83). The first approach is being further developed in the FP6 project, DetecTox (61).

**Conclusions concerning biochemical/immunological methods**

From the review of currently available methods for marine biotoxins, it is clear that very few have been sufficiently validated to serve as reference methods in cases where there is dispute. However, a number of methods are very well advanced and can serve as screening methods, in the sense of the distinction between screening and confirmatory methods made (84). This distinction may also be particularly important when innovative legislative strategies are considered to reduce the use of animal tests rapidly (see the Acceptance of Non-Animal Based Reference Methods section, below). Therefore, it is highly recommended that these methods are also considered for further collaborative validation, to increase their acceptance as official screening methods.

**Chemical analytical methods**

Chemical analytical methods are typically based on liquid chromatography (LC) to separate a crude or clean extract, followed by toxin-specific detection by using physicochemical methods, for example by UV (LC-UV), fluorescence (LC-FL) or mass spectrometry (LC-MS). Due to the compound-specific detection of these methods, the raw data obtained relate to individual concentrations of the compounds pres-
ent and must be transformed into toxic equivalents by using conversion factors.

**Azaspiracid (AZA) group**
AZAs lack a chromophore for LC-UV determination, and the conditions for LC-FL measurement have not been established. However, LC-MS has shown great promise as a highly specific and sensitive technique for the detection of AZAs. One multi-toxin protocol has been subjected to a full single-laboratory validation (four shellfish species) and a limited interlaboratory study (85). The limit of quantification (LOQ) for this method was 0.05mg/kg, but lower limits would be readily achievable, which will be necessary to achieve the levels proposed by the FAO/WHO/IOC experts (2). Because an LC-MS method is the best available option, a collaborative study should be conducted on a multi-toxin method that includes AZAs. However, the applicability of the method is currently limited by the lack of certified analytical standards.

**Brevetoxin group**
LC-MS methods have been developed to detect a wide range of brevetoxins. However, validation has been carried out for only a few toxins for which analytical standards were available. Good recoveries and precision, and LOQs of 0.03mg/kg have been reported for several brevetoxin metabolites. LC-MS testing for these toxins as markers of brevetoxin contamination has been recommended. However, further method development and validation is required for LC-MS methods that quantitatively determine a wider range of toxicologically relevant metabolites. This would permit the regulation of brevetoxin contamination in shellfish. No method currently meets the criteria for a reference method. A collaborative study is needed on an LC-MS method or a functional method that detects a range of the toxicologically relevant brevetoxin metabolites.

**Cyclic Imines group**
LC-MS methods have also been developed for gymnodimine and spirolides, which are suitable for screening and for confirmation. One multi-toxin method that includes gymnodimine has received a full single-laboratory validation and a limited interlaboratory study (85). A small interlaboratory study on spirolides in algal extracts has also been completed.

**Domoeic Acid (DA) group**
LC-UV is the current basis for the regulatory testing of the DA group. The existing AOAC method (86) has been collaboratively studied and used for many years for regulatory purposes (87). However, a more recent method (68) yields more stable extracts and is preferred. This method has undergone two successful interlaboratory trials and proficiency testing (88, 89). One LC-MS method (90) has received a full single-laboratory validation. A thin layer chromatography (TLC) method is available as an inexpensive screening test (91).

**Okadaic Acid (OA) group**
An LC-FL method (92) has been used in several laboratories with good results. The method has been validated for OA in mussel digestive glands in an interlaboratory study, and the results have been submitted to the European Standardisation Committee (Comité Européen de Normalisation [CEN]) for approval. Validation data are lacking for DTX1 and DTX2, but the method has also been used for these analogues. One LC-MS method (85) has undergone a full single-laboratory validation and a limited interlaboratory study.

**Pectenotoxins (PTX) group**
LC-MS methods have been developed for PTXs, which are suitable for screening and for confirmation testing. One multi-toxin method has been subjected to a full single-laboratory validation and a limited interlaboratory study (85). Good recovery data have been reported for PTX1, PTX2 and PTX6 from scallop tissues.

**Saxitoxin (STX) group**
A pre-chromatographic oxidation LC-FL method (93) has been collaboratively studied, and was approved by AOAC International in June 2005 (94, 95). Its approval by the CEN is pending. The correlation with MBA data was high. A post-column LC-FL method (96) is widely used, but has not been the subject of a full interlaboratory study. Correlations with MBA data have given favourable results. Other instrumental methods, such as LC-MS, are at an early stage of development.

**Yessotoxin (YTX) group**
A derivatisation LC-FL method (97) has been routinely used in several laboratories for the YTX group, homoYTX and the 45OH metabolites. It cannot detect carboxy-YTX and other analogues lacking the conjugated diene. Further validation data are needed, as is an interlaboratory study. One multi-toxin LC-MS method study (85) has received a full single-laboratory validation and a limited interlaboratory validation. Further interlaboratory studies are needed.

**Conclusions concerning analytical methods**
From the review of currently available analytical methods for marine biotoxins, it is clear that very few have been sufficiently validated for use as replacements to the currently-used animal tests, especially for enforcement purposes or for reference methods in cases where there is dispute. Only the methods for DA and the STX group have been extensively evaluated in interlaboratory studies and can be recommended immediately. It is strongly
recommended that candidate instrumental methods be identified and submitted to validation studies to overcome this situation for all toxin groups. Table 2 lists instrumental approaches and other types of methods available that could provide alternatives to animal tests, once they have been successfully validated according to internationally accepted criteria.

**General conclusions on available animal and non-animal methods**

1. Animal use authorised to protect man and the environment under the provisions of Council Directive 86/609/EEC (10) must reflect our current state of knowledge, taking account of the technical progress that has been made.

2. Given the nature of the MBA used to detect, identify and quantify marine biotoxins — a non-validated procedure, with known and unknown interferences, which can entail the use of large numbers of animals and inflicts substantial levels of animal suffering — and the progress made on reduction, refinement and replacement of the MBA in Europe and elsewhere, it is timely to consider specifically whether our current state of knowledge permits the quality of science to be increased and the animal suffering caused eliminated or decreased by using these alternative methods.

3. The majority of the participants in the workshop endorse the conclusions of the FAO/IOC/WHO Expert Consultation group on marine biotoxins (Table 2 [2]), which suggest that animal-based methods are not appropriate reference tests for marine biotoxin testing, mainly for the scientific reasons that the appropriate MRLs cannot be implemented with existing animal tests, due to interference. A review of some of these limits, due to new information on the amount of shellfish consumed in a given meal, is still in progress. As agreed during the last CODEX Alimentarius meeting (Cape Town, South Africa, 28 February–4

<table>
<thead>
<tr>
<th>Toxin group</th>
<th>Functional method</th>
<th>Biochemical/ immunological method</th>
<th>Analytical method</th>
<th>Animal test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azaspiracid</td>
<td>Cell morphology</td>
<td>NA</td>
<td>LC-MS(^a)</td>
<td>MBA/RBA</td>
</tr>
<tr>
<td>Brevetoxin</td>
<td>Na-channel receptor binding assay; neuroblastoma</td>
<td>ELISA</td>
<td>LC-MS(^a)</td>
<td>APHA-MBA</td>
</tr>
<tr>
<td>Cyclic imine</td>
<td>NA</td>
<td>NA</td>
<td>LC-MS(^a)</td>
<td>MBA</td>
</tr>
<tr>
<td>Domoic acid</td>
<td>Receptor-binding assay</td>
<td>ELISA, immunobiosensor</td>
<td>LC-UV(^b), LC-FL, LC-MS, TLC</td>
<td>NA</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>PP2A, PP1, F-actin</td>
<td>ELISA</td>
<td>LC-MS(^a), LC-FL</td>
<td>(S)MBA/RBA</td>
</tr>
<tr>
<td>Pectenotoxin</td>
<td>F-actin</td>
<td>NA</td>
<td>LC-MS(^a), LC-FL, LC-UV</td>
<td>MBA</td>
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<td>Na-channel receptor binding assay; saxiphilin receptor binding assay; neuroblastoma</td>
<td>ELISA, LFIC FIFLD</td>
<td>LC-FL(^b), LC-MS, AOAC-MBA</td>
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<td>Yessotoxin</td>
<td>E-cadherin fragmentation; PDE-enhancement</td>
<td>ELISA</td>
<td>LC-MS, LC-FL MBA</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)recommended as a reference method after completion of successful collaborative trial; \(^b\)recommended as a reference method; NA = not applicable; MBA = mouse bioassay; RBA = rat bioassay; AOAC-MBA = mouse bioassay according to AOAC International (19); APHA-MBA = American Public Health Association mouse bioassay; PP2A = protein phosphatase 2A; PP1 = protein phosphatase 1; F-actin = filamentous actin; PDE = phosphodiesterase; ELISA = enzyme-linked immunosorbent assay; LFIC = lateral flow immuno-chromatography; LC-FL = liquid chromatography with fluorescence detection; LC-MS = liquid chromatography with mass spectrometric detection; LC-UV = liquid chromatography with ultraviolet absorption detection; TLC = thin layer chromatography; FIFLD = flow injection fluorescence detection.
March 2005), the establishment of a working group to address this subject is foreseen, which should convene in Canada in 2006.

Tools and Stakeholders in Validation and Standardisation

Reference standards and tissue materials

Reference materials, and in particular certified reference materials, play an increasingly important role in analytical quality assurance. The following definition of a certified reference material (CRM) is used in International Standards Organisation (ISO) terminology: “Certified Reference Material — a reference material, accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes its traceability to an accurate realisation of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence” (98). In simpler jargon, CRMs for marine biotoxins are stable, homogeneous products, with certified value(s) for the toxin(s) of interest. CRMs can be used for several purposes (99):

— for calibration, i.e. calibrant (C)RMs, for identification and quantification;

— in regular analyses, as part of AQA programmes, i.e. matrix (C)RMs, in control charts; and

— in method development and validation, i.e. calibrant and matrix (C)RMs, for comparison of methods and determination of bias.

Conclusions

Various international organisations (FAO, AOAC International, and EU-NRL network on marine biotoxins) have acknowledged the need for (C)RMs for marine biotoxins. There are several essential requirements for marine toxin (C)RMs. They must be:

— analytically relevant, i.e. the toxin(s) should be present in the natural state, and at “normal” concentrations;

— homogeneous within the variability of methods applied in practice;

— stable over a longer period of time, i.e. for several years;

— practical in terms of packing, storage and distribution; and

— accompanied by documentation, justifying the certified value and its uncertainty.

Homogeneity investigations serve to verify unit homogeneity both between and within units. For the analytical method, precision is important, and measurements have to be carried out under repeatability conditions. For blank samples, a simplified procedure can sometimes be used, as the purpose here is to demonstrate the absence of the toxin(s). The results of the homogeneity investigations can often serve as the start of the stability investigations. The latter are needed to demonstrate long-term stability and to establish expiry dates, where applicable. Stability investigations may focus, in particular, on the effects of special treatments (e.g. lyophilisation, pasteurisation, canning), and the possible influence of packing circumstances (e.g. inert gas, container material, laminate sachets). Here also, the precision of the analytical method is important, and measurements have to be carried out under repeatability conditions as much as possible, which can be achieved by carrying out isochronous measurements. Stability investigations need to be carried out at various temperature–time combinations, to be able to detect potential instability at an early stage, and to establish the best storage and shipment conditions. Increased temperatures are used to accelerate decomposition, which otherwise may be difficult to detect over relative short periods of time. As with the homogeneity investigations, blank samples may sometimes undergo a simplified procedure.

The undisputed world authority in the area of CRMs for marine biotoxins is National Research Council (NRC) Canada, which began producing toxin standards in response to the DA crisis in Canada in 1987. Since then, the programme has expanded to include calibration solution CRMs and (a few) shellfish tissue CRMs for a variety of toxins of marine algal origin, including those responsible for amnesic, diarrheic and paralytic shellfish poisoning (100). NRC Canada bases certified values on measurements obtained by at least two independent analytical methods.

Another certifying body, which has CRMs for marine biotoxins, is the Institute for Reference Materials and Measurements (IRMM) of the European Commission’s Joint Research Centre (JRC). Lyophilised mussel tissue CRMs, certified for their STX and dc-STX contents, were developed in the 1990s within the EC’s Measurements and Testing Programme, and were registered as BCR CRMs 542 and 543 ([101]; N.B. the BCR, the Bureau Communautaire de Référence, no longer exists). BCR materials are certified according to very strict guidelines (102), and the certified values are based on interlaboratory studies, usually involving 10–15 partners, followed by evaluation and approval by an independent committee. Unfort-
Unfortunately, the distribution of the BCR CRMs is severely hampered by the fact that STX is a schedule 1 chemical on the list of the Chemical Weapons Convention.

In addition to these certified CRMs, other reference materials for marine biotoxins are available from various sources. Some of these are quality controlled and have already been supplied for several decades, such as the STX standard solutions from the US Food and Drug Administration (FDA). Finally, some other test materials and solutions are (rather scarcely) available from various sources, with different levels of characterisation and quality. These are often in private collections, and are useful for method development, qualitative purposes, in-house studies and proficiency tests.

In the EU, where legislation for marine biotoxins is quite diverse and detailed, there is a strong need for reference materials. Although the CRMs of NRC Canada are available (as calibrant solutions) for many of the regulated compounds, tissue reference materials remain rather scarce. Moreover, all CRMs are expensive, due to the extensive work involved in their development, and the supplies are limited. Various activities are being undertaken in the EU to improve this situation, as witnessed by the ambitions of the FP6-sponsored projects, BIOTOX (58), BIOTOXMarin (103), DetecTox (61) and BioCop (82), all of which started in 2005. All these projects include plans to isolate toxins for research purposes. OA, DTX, YTX, PTX, AZA and PSP toxins are on the wish lists. An additional target of the BIOTOX project is the conduct of feasibility studies on the production of matrix CRMs for some selected lipophilic toxins of European interest (e.g. YTXs, AZAs). Apart from these EC-sponsored collaborative projects, some individual institutes have undertaken initiatives to develop materials for instrument calibration, as well as further toxicology studies, such as for AZAs (104).

Despite these developments, the recent FAO/WHO/IOC Expert Consultation group (2) concluded that “All areas of marine biotoxin method developments, validation and testing require certified calibration standards and reference materials. There is an urgent need to expand the currently available CRMs and the Codex Alimentarius should encourage Member States to fund the necessary efforts”.

The Association of Official Analytical Chemists (AOAC) International and AOAC Official Method Validation

AOAC International is an international analytical methods validation organisation that has been in existence for well over 100 years. It was created as a part of the US FDA, but it is now an independent not-for-profit association. Many national and international organisations and regulatory agencies around the world rely on AOAC validated methods for carrying out their work; for example, many countries refer to AOAC methods in their official legislation for mycotoxins (105). AOAC International validates the performance characteristics and fitness for purpose of analytical methods based on strict internationally accepted criteria, including harmonised protocols for interlaboratory collaborative studies (106) and single laboratory method validation (107). The Association makes use of independent scientific experts for the review and evaluation of submitted methods, through various methods committees. Methods for marine biotoxins are handled by the Methods Committee on Natural Toxins and Food Allergens.

AOAC International distinguishes three types of validation:

1. **Single Laboratory Validation** (carried out in a single laboratory according to a rigorous scheme, to ensure that “off-the-shelf” validated methods are being used correctly).

2. **Peer Verified Validation** (a method developed in one laboratory and tested in one or two other laboratories, and evaluated against specific standards).

3. **Full Collaborative Study** (the independent testing of a method by at least eight different laboratories submitting valid data for a quantitative method, and at least 15 different laboratories submitting valid data for a qualitative method. The minimum number of test materials is five).

AOAC International has recently created and implemented the concept of “communities”, to assist with identifying those methods that are needed to serve the community; for example, there is now established a Marine and Freshwater Toxin Community, consisting of over 150 international members from government, academia and industry. Expert review committees are established within each community, to identify and prioritise those methods that should go through the AOAC International method validation process. Generally, the validation process includes:

- a Single Laboratory Validation (SLV);
- the Peer Verification of the results of the SLV study;
- a Full Collaborative (FC) Study;
- the Peer Verification of the results of the FC study;
- acceptance as an “AOAC Official First Action” method; and
Although much progress has been made in marine and freshwater toxin research, currently only three AOAC Official Methods exist for marine biotoxins: an MBA for PSP toxins (19), an HPLC method for DA (87), and a further HPLC method for the STX group (95). To stimulate the provision of new methods in the “Official Methods of Analysis” that meet the needs of the stakeholders, in 2003, Jim Hungerford, the AOAC General Referee for Marine and Freshwater Toxins, has proposed the establishment of a new analytical community, The Task Force on Marine and Freshwater Toxins. The Task Force’s Terms of Reference were approved in June 2004 (James Lawrence, personal communication) and its responsibilities include:

— serving as experts in the field;
— developing and prioritising a list of marine and freshwater toxins that need validated methods;
— assisting in identifying existing methods for validation through AOAC validation programmes;
— helping in identifying sources of funding for validation studies;
— identifying potential participating laboratories and samples;
— increasing programme awareness among stakeholders;
— assisting AOAC International in identifying study directors; and
— identifying and developing other quality measurement tools (training).

Task Force membership has grown rapidly since its inception, with international representation from universities, government agencies, and industry groups. Among the first achievements of the Task Force were the establishment of a well-balanced voting group (12 members) and the development of criteria for prioritising methods to be addressed by the Task Force. A major criterion for prioritisation is the demonstrated need for a method (for example, because of human illness, economic damage, regulatory requirements, bioterrorist potential). Other important factors include the priorities indicated by other groups (e.g. Codex Alimentarius, UNESCO, FAO, EC, CEN, FDA), and the possibility of providing alternatives to animal testing (ECVAM). Finally, the existence of a full AOAC method performance interlaboratory study would be an asset. The Task Force also developed criteria for selecting methods for validation and for guiding method development. These criteria refer to method performance and practicality. Method performance characteristics include information about whether a method’s response to an analyte is related to toxin levels or activities, dynamic range, limit of determination, recovery, AOAC single laboratory validation, ruggedness, and availability of standards. Practicality is equally important, and involves issues such as sample throughput and the availability of materials and equipment. In 2004, the voting members of the Task Force voted positively about two methods which had been the subject of collaborative studies, a non-proprietary LC-method for PSP toxins (93, 94), and a proprietary ELISA for DA (69). The first method has already been approved by AOAC International (95), while the second method is still undergoing technical review by AOAC’s Methods Committee on Natural Toxins and Food Allergens. The Task Force has the ambition to review and vote about two more methods in 2006.

The European Standardisation Committee (CEN) and Method Standardisation

CEN is the European equivalent of the ISO, and comprises members of national standardisation bodies from European countries (not exclusively EU MSs). CEN produces standard methods to enable food manufacturers to determine, with reasonable certainty, whether a production batch may be put on the market, and to enable regulatory authorities to determine whether foodstuffs on the market comply with legal limits. CEN standards are in principle voluntary standards, but the EC sometimes refers to CEN standards for substances subject to legal limits. Within CEN, Working Group 5 (biotoxins) of Technical Committee 275 (Food Analysis-Horizontal Methods) has worked on the selection and elaboration of methods for mycotoxins (since 1993) and marine biotoxins (since 1999), that are to become EU standards. The WG 5 subgroup on marine biotoxins works closely with the EU NRLs on Marine Biotoxins.

There are various steps in the standardisation process, before a method reaches the status of a CEN standard. After the need for a method is identified, selection by the WG takes place. In principle, only interlaboratory-validated methods with broad applicability are selected. Then a project leader is nominated, who is charged with drafting the method in ISO/CEN format, which includes a summary of the performance characteristics. Discussion takes place in the WG followed by amendments, where necessary, and WG approval. The draft is then distributed among CEN member states, to gather comments. The comments are discussed in detail in the WG, usually leading to further improvement and editing of the method descrip-
tion. Then the final draft is sent for formal (weighted) vote by CEN member states. If approved (71% or more of the weighted votes cast are in favour), the standard is transposed into a national standard. Conflicting national standards must be withdrawn. CEN standards are published in English, French and German.

At the time of writing, several methods of analysis for marine biotoxins have been standardised by CEN WG 5. These are:

— an LC method with UV detection, for DA in mussels (108);

— an LC-method with pre-column derivatisation followed by fluorescence detection, for OA in mussels (109);

— an LC-method with pre-column derivatisation followed by fluorescence detection, for STX and dc-STX in mussels (110); and

— an LC-method with post-column derivatisation followed by fluorescence detection, for OA in mussels (111).

All these methods are based on chromatographic techniques. In principle, standardised CEN methods can also be based on other techniques, e.g. receptor-based assays or biosensor technology. However, CEN does not standardise animal tests for marine biotoxins, such as the MBA currently prescribed in EU legislation for PSP toxins, DSP toxins and some other lipophilic toxins (112).

The methods selected for standardisation by CEN WG 5 must be of proven quality and acceptable performance in interlaboratory studies. Ideally, these methods should have undergone a full collaborative study according to the “harmonised” protocol (106). However, fully validated methods for marine biotoxins are currently scarce, whereas the need for a standardised method may exist. Therefore, data from other types of interlaboratory studies (e.g. certification exercises, proficiency studies) may be suitable, if acceptable interlaboratory precision data can be derived.

The evaluation and acceptance of these interlaboratory performance characteristics is carried out by CEN WG 5. Currently this is based on expert experience and opinions, but the working group is drafting a document with performance criteria of phycotoxin methods, that can be used for this purpose in the future. This is in analogy with the production by WG 5 of a similar document on performance criteria for mycotoxin methods, published in 1999 (113). The latter has proven to be very useful, and parts of its contents can be found in the EU harmonised legislation for mycotoxins. The current draft document on criteria for phycotoxin methodology now includes limited perform-

The European Centre for Validation of Alternative Methods (ECVAM)

ECVAM was created by a Communication from the EC to the European Council and the European Parliament in October 1991 (115), pointing to a requirement in Directive 86/609/EEC (10) on the protection of animals used for experimental and other scientific purposes, which requires that the EC and the MSs should actively support the development, validation and acceptance of methods which could reduce, refine or replace the use of laboratory animals. ECVAM was established in 1992 as a unit within the EC’s JRC, and is now part of the JRC Institute for Health and Consumer Protection. ECVAM’s main task is the coordination of the requirements for in vivo and in vitro methods and safety assessment, and the coordination of the development of in vivo and in vitro test methods.
and funding of validation studies on non-animal test methods for regulatory purposes, i.e. methods to replace animal tests in the current legislation for the safety testing of chemicals, cosmetics, pharmaceuticals and other chemical products.

In 1995, in cooperation with international experts and based on the experience gained in validation studies, ECVAM established a formal process for the prospective validation of alternative methods (116, 117). An alternative method can be thought of as a combination of a test system and a prediction model (118, 119), where the prediction model is an algorithm which converts the data generated with the in vitro test system into a prediction of a toxicological endpoint in animals or humans. Both are independently assessed during a validation study. Comprehensive reviews of ECVAM’s work from 1993 to 2002, the validated and accepted in vitro methods, and the challenges posed by EU policies on chemicals and cosmetics were recently published (120–122).

In order to make the validation process more flexible for already widely-used methods and more applicable for new technologies, ECVAM recently published proposals for a modular approach of validation, which would permit a combination of prospective and retrospective validation (123).

**Recommendation**

Due to its unique experience in the validation of methods to replace animal tests in the regulatory framework, ECVAM’s advice should be sought for the validation of methods to replace the in vivo methods for marine biotoxin testing.

**Research Requirements for the Validation of Non-Animal Based Methods**

The acceptance of alternatives to animal-based methods for biotoxin detection demands that estimates are both accurate in terms of the amounts measured for individual compounds and significant with regard to the overall toxicity of samples. Thus, a consensus on procedures for intercomparison of results obtained by different methods would greatly facilitate any acceptance of alternatives.

Three concepts have been developed for the rational quantification of the relevant burden of toxins in field samples and for comparisons among estimates obtained by different methods. These include the Toxicity Equivalence Factor (TEF) concept, developed in the field of functional methods, as well as the Marker Compound (MC) and Relative Response Factor (RRF) concepts, particularly to be applied in the field of chemical analytical methods.

**The Toxic Equivalence Factor (TEF) concept**

Definition: TEFs represent the potency of an analogue as compared to that of a reference compound. A simple way to express the TEF of a toxin analogue is by the ratio between the EC50 of the reference compound and that of the analogue itself. The value can then be used as a multiplier of the absolute concentration of that analogue, yielding the estimate in toxin equivalents of the reference compound.

The simple addition of the equivalents calculated for the different analogues, as detected by analysis of the individual components, has often been considered to represent the overall content of biologically active toxins present in field samples. However, the additivity should be experimentally demonstrated to occur in the system chosen for the analysis of toxins. When a receptor component is involved in the mechanism of action of toxins, for instance, the response is a hyperbolic function of toxin concentrations, and simple additivity might not apply.

The use of analytical methods for the determination of the overall toxicity present in a sample is particularly appropriate for the assessment of the risk associated with the panel of individual toxins detectable in any specific material. Therefore, the comparison of the performance of functional methods with reference to chemical analyses for the purpose of cross-technique validation or acceptance demands that TEFs of toxicologically relevant analogues for each toxin group are known, and that the mathematical treatment of experimental data is agreed upon.

Currently, TEFs for marine biotoxins are not defined in EU legislation, so confusion exists as to whether the TEFs should be based on comparative toxicities as observed in animal tests or based on toxicities estimated from functional methods. While, ideally, the toxicities from animal models and those from truly functional methods should be the same, little research has been carried out to demonstrate that this is true for some groups of marine biotoxins. There is a potential problem with animal-test based TEFs, if the animal tests are conducted by using i.p. injection, i.e. EC50 = LD50, since this mode of administration neglects the fact that the uptake route for shellfish in humans is different. However, this is also the general problem of the currently applied animal-based methods, which needs to be investigated in the context of the general toxicities of the toxins compared to epidemiological data.

Most progress on comparative toxicities has been made in the area of STXs, where comparative MBA toxicities published by Oshima (124) are also well correlated with the comparative binding activities of STX analogues in the receptor binding assay (125). Comparative toxicities have also been determined by
i.p. injection into mice for OA (126), DTX-1 (127), DTX-3 (128), AZA-1 (129), AZA-2 and -3 (130), PTX-1 and PTX-2 (128), and YTX (131, 132), OH-YTX, homo-YTX and OH-homo-YTX (133–135). Research is in progress at the Marine Institute and the Norwegian School of Veterinary Science, to determine the comparative toxicities of DTX-2 both in mouse i.p. injection studies and in the PP2A assay.

Recommendation

Due to the urgency of the need to replace animal-based methods in this area, the TEF concept should be agreed upon, and the most appropriate factors and mathematical treatments of data to be used should be determined. For this purpose, DG SANCO should convene a meeting involving international experts and stakeholders.

The Marker Compound (MC) and Relative Response Factor (RRF) concepts

These concepts were developed in the framework of the AOAC International task force by Holland and McNabb during 2004 (Philipp Hess, personal communication), and were used as an internal task force discussion paper. The following summarises their main aspects.

Background to the MC and RRF concepts

The need for transition to instrumental analysis and immunoassays from animal bioassays for marine biotoxin testing of seafood products, has brought to the fore issues relating to the identification of the toxic residues to be regulated, setting regulatory limits, establishing suitable analytical methods for enforcement, and the calibration of the assays.

The range of toxins produced by hazardous algae is generally complex, and varies by species and location. The metabolism of the toxins in shellfish can lead to the enhancement or reduction of their toxicity to mammals, and the paths and rates for this metabolism are dependent on the shellfish species concerned. Overall, these factors can lead to complex and often poorly defined mixtures of toxins and their derivatives in contaminated shellfish samples. For example, YTXs are currently regulated by the EU and New Zealand at 1mg/kg for YTX plus its 1-homo analogue and their respective 45-hydroxy metabolites (45-OHYTX). However, recent research has shown that: a) the acute toxicity of YTX by the oral route is low; b) other YTX analogues are produced by the contaminating alga, Protoceratium reticulatum; c) other significant metabolites are formed in shellfish, including carboxyYTX; and d) a wider range of immunoreactive forms of YTX are present in the algae and in contaminated shellfish. Currently, certified standard material is only available for YTX (100).

Similar issues with regard to the definition of toxic residues and analytical methods for the regulation of set limits have had to be addressed in the regulation of other contaminants in food, particularly mycotoxins, pesticides and veterinary drugs. The concept of a marker residue has been elaborated to permit the regulation of contaminants in food, where it is not practical on a routine basis to fully determine the levels of all residues present. The levels of the marker in a food sample can be used to estimate the total toxic residues present, and to decide whether a regulatory limit has been exceeded.

A related issue is the use of relative response factors (RRFs) for the calibration of instrumental assays for particular toxins, where certified reference standards are not available. In the above-mentioned discussion paper of the AOAC International task force, the definitions, practicality and limitations in the use of marker compounds and RRFs are examined in the context of analysis for marine biotoxins in shellfish.

Marker compounds

Definition of Marker Residue (veterinary drug): Residue whose concentration is in a known relationship to the concentration of the total residue in the last tissue to deplete to its permitted concentration. The marker residue can be the sponsored compound, any of its metabolites, or a combination of the residues for which a common assay can be developed. The target tissue and marker residue are selected so that the absence of marker residue above a designated concentration R(M) will confirm that each edible tissue has a concentration of total residue at or below its permitted concentration. (136)

For veterinary drug residues in animal products, the application of the concepts of marker residue and target tissue requires an experimental determination of the quantitative relationships among the residues that might serve as the marker residue in each of the various edible tissues that might serve as the target tissue. Because these relationships may change with time, the depletion of potential marker residues in potential target tissues must be measured, starting after the last treatment with the drug and continuing until the residue has reached the permitted concentration for that tissue. The sponsor may use the results from the total residue depletion and metabolism studies carried out with the radio-labelled drug to determine the marker residue, the target tissue, and the R(M).
Marine biotoxins are regulated on the basis of risks to human health — concepts such as Good Agricultural Practice do not apply, which can lead to the regulation of veterinary drugs or pesticides as “no detectable residues” or at other levels below limits based solely on safety. Thus, for use in managing health risks from shellfish, the marker compound and its R(M) must have a demonstrated relationship to the regulatory limit, however they are expressed. Other differences and limitations that affect the application of the marker compound approach to the regulatory testing of marine biotoxins in shellfish include the following:

1. Hazardous algae generally produce complex mixtures of marine biotoxins that vary even within species. Shellfish are exposed to mixtures of toxins, including minor analogues, some of which may not have been characterised or taken into account when setting regulatory limits.

2. Experimental studies using radio-labelled toxins are generally not available as a basis for the accurate determination of metabolism and depuration kinetics. Even data from non-labelled studies may be limited in quantity, cover only one or two shellfish species, and be largely obtained from field exposures with associated uncertainties.

3. Toxicological studies on marine biotoxins are often very limited in scope and precision when compared to those required to be carried out for new drugs or pesticides. This is due to the limited range and quantities of purified toxins and their metabolites that can be produced and the expense of the associated toxicology. Often, only acute toxicity data in mice or rats are available. This paucity of reliable and comprehensive data leads to major difficulties in the setting of regulatory limits that are securely related to risks from the human consumption of contaminated shellfish. The uncertainty in the quantitative relationship between the levels of a marker compound and the potential human toxicity of contaminated shellfish samples, includes a major component from the uncertainty of the evaluation of the toxicity of the compound in the first place.

These factors make it difficult to establish a quantitative relationship between the levels of a nominated marker and those of all the toxicologically relevant compounds in shellfish tissues. Even for DSP toxins, for which the EU regulatory limit is 0.16mg/kg, it is not possible to use OA as a quantitative marker for total DSP (OA, DTX1, DTX2 and their esters), due to large variations in the profiles of these toxins produced by algae and their accumulation in shellfish (species/time). It is important to note that the regulatory limits for classes of toxins set using levels of individual compounds, such as for DSP, already embrace the MC concept, because the limits implicitly include a range of other related metabolites and analogues that are not measured in enforcing the limits; i.e. the marker for DSP toxicity is the sum of OA, DTX1 and DTX2 in hydrolysed samples. However, the situations where a MC that does not comprise the major proportion of the overall toxicity could be used to directly manage human risk, are likely to be limited to a few very well-researched scenarios, for example Karenia brevis contamination of Eastern oyster with brevetoxins.

An alternative scenario, not envisaged in the veterinary drug definition, is the use of MCs to screen for the presence or absence of particular toxin classes. A positive result for the screening test would require an alternative quantitative test, to determine whether the sample exceeded the regulatory limit. In the absence of a known relationship between level of marker and total regulated residue, it will be sufficient to show that the limit of detection of the screening method for the MC is low enough to avoid false negatives; i.e. all samples containing total residues above the regulated limit would be detected. This screening approach can be particularly useful where instrumental analyses are being used to test shellfish regularly, where the majority of samples are expected to test negative, e.g. as part of large monitoring programmes. The following examples show where this screening concept could be useful in the application of LC-MS analyses to regulatory testing:

1. **DSP toxin esters/DTX3. Dinophysis** species produce OA, DTX1 and DTX2 in proportions that vary widely according to species and location. Other isomers may be present, and a significant proportion of the toxins may be in esterified forms. Further transformations with respect to esterification take place after ingestion by shellfish. Shellfish contaminated by DSP toxins contain free toxins and a proportion in ester forms such as DTX3, which must be released by hydrolysis to evaluate overall compliance with the regulatory limit. Certified standards are only available for OA, although a CRM for mussel tissues containing both OA and DTX1 is also available. Contamination of shellfish by DSP toxins is almost exclusively caused by Dinophysis species, which also produce much higher levels of PTXs, mainly PTX2. The rapid hydrolysis of PTX2 in shellfish leads to the production of PTX2 seco acid, which can be sensitively detected by LC-MS. The use of PTX2 seco acid as a MC for the onset of DSP toxin contamination permits the LC-MS screening of shellfish by using a single run per sample; a second run...
of the hydrolysed sample is only required when PTX2 seco acid is detected.

2. Brevetoxins/neurotoxic shellfish poisoning (NSP) toxin. The spectrum of brevetoxins produced by Karenia brevis is dominated by brevetoxin 2 (PbTx-2), but a range of other brevetoxins are generally present at lower levels. Brevetoxins undergo a complex metabolism in shellfish, with oxidations, reductions and conjugations of the terminal side-chain leading to a wide range of derivatives, many of them toxic. Certified standards are available for PbTx-1, PbTx-2, PbTx-3 and PbTx-9, but not for brevetoxin B5 or any of the conjugates which dominate the toxic residues in contaminated shellfish. Parent PbTx-2 and the minor parent toxin and primary metabolite PbTx-3, have been shown to be present in shellfish contaminated by Karenia species. Although the levels of PbTx-2 and PbTx-3 are relatively low, the limit of detection of LC-MS is such that samples contaminated at levels well below the regulatory limit for NSP toxin are readily detected. Thus LC-MS screening for NSP toxin involving these MCs can reduce the amount of mouse testing according to the standard APHAX-ether protocol, without compromising public safety.

3. AZAs. AZAs can be readily detected in shellfish by using LC-MS. However, they are a complex class of toxins, for which three major analogues and a range of minor analogues are known. They are also relatively rare internationally. A standard is available for AZA-1, so it is convenient in the multi-toxin screening of shellfish to set-up the LC-MS method to detect this toxin. In the event of detection of this MC, samples can be re-run by LC-MS or another appropriate technique, in order to determine a fuller range of the AZAs present, so that the regulatory limit can be enforced.

Conclusions
The use of MCs to provide quantitative estimates of the total toxic residue has limited application in the direct enforcement of regulatory limits for marine biotoxins in shellfish, due to the highly variable and complex nature of shellfish contaminations. However, MCs are very useful for screening purposes, especially in routine monitoring programmes, where they can reduce the complexity and cost of the testing, while also protecting public safety.

RRFs
Definition of Relative Response Factor: Ratio of instrument responses (generally peak areas for chro-

matographic methods) for a compound of interest to that of a reference compound at equal concentration, or the ratio of the slopes of the linear calibration equations. The concentrations of the compound of interest in unknown samples can be estimated following application of the previously established RRF to the calibration equation established for a reference compound.

RRFs are useful for the calibration of instrumental assays for particular toxins, where certified reference standards are not available for routine use. Two situations can be recognised:

1. Only small quantities of a quantitative standard are available, but they are sufficient to establish the RRF to a reference toxin in a few instrument runs. This is a very viable approach, but the accuracy of the RRF is dependent on the accuracy of the concentration of the standard, which is likely to be poor for small quantities of relatively rare toxins. The precision of the RRF can be checked by re-determination at intervals, if sufficient quantities of the standard are available in a stable form.

2. No quantitative standard is available. This is the situation for many toxins and their metabolites, although crude standards or contaminated shellfish extracts are generally available for establishing retention times and spectroscopic characteristics. The application of a RRF must then rely on chemical similarities between the toxin and chosen reference compound, such that an assumption of equimolar responses, i.e. RRF = 1, can be taken as a reasonable approximation.

RRFs should only be applied where the toxin and its reference have close chemical similarity and behaviour in the analytical system; i.e. when there are minimal differences in detector response factors and small differences in retention times. Generally, the concept has been applied where a parent toxin such as OA or YTX is available as a certified standard, and an assumed RRF of 1 is taken for an isomeric toxin or a simple analogue or metabolite, e.g. DTX1 (35-methyl OA) or 45-OH YTX. This assumption should be valid, if the structural changes are minor and not close to the chromophore, where the detection system is spectroscopic (UV or fluorescence); for example, the HPLC-FL methods for OA, DTX1 and DTX2, and for YTX and 45-OH YTX, which are calibrated by using OA and YTX, respectively.

The application of RRFs to LC-MS methods is more problematic, because electrospray ionisation efficiency is highly dependent on chemical structure and the ionising environment (solvent composition; coeluting coextractives). A good example of the
establishment of RRFs is the study by Pleaseance et al. (137), establishing RRFs for OA, DTX-1 and DTX-2 by comparison of an LC-MS method with an LC-FL method. It is more difficult a priori to justify the assumption of equal response factors. This may be a good approximation in groups of large polyether toxins, where ionisation can be assumed to be mainly at a common moiety relatively remote from the structural changes, for example the positive electrospray ionisation (ESI) of AZAs with the common ionisable nitrogen-centre, or of negative-ESI of YTXs with the common electronegative sulphate groups. However, other subtle effects on ionisation efficiency may arise from changes in properties such as surface tension in the electrospray droplets. There are also likely to be shifts in an RRF, in moving from the selected ion recording (SIR) of molecular species, for example, positive (MH+) or negative ([M-H]-) molecular ion to tandem MS (MS/MS) with multiple reaction monitoring (MRM), due to the effects of structure on the relative intensities of the key ions in the collisional activation spectra. However, the RRF can be corrected for these changes by comparing the responses of the toxin and the reference, by using SIR and MRM. Absolute concentrations are not required, so crude standards or contaminated shellfish are adequate for this determination.

The advantages of RRFs are that concentration data can be obtained for a wide range of toxins and analogues in shellfish, based on a limited set of standards for the parent toxins and suitably contaminated shellfish extracts. Precision data can be gathered and general analytical Quality Assurance and Quality Control can be maintained. Although the use of RRFs increases the uncertainty of a determination, the data sets are consistent, and trends can be accurately followed. For monitoring programmes, these are significant advantages, and public health risk can be assured by reference to a relatively limited amount of additional testing, e.g. by the MBA. Uncertainties arising from the use of these instrumental RRFs will generally be less than those in the toxicological data used to establish the No Observable Effect Levels, and will generally be covered within the safety factors, especially where the proportion of analogues is relatively low. The use of RRFs is accepted in other natural toxin areas, such as in testing for fumonisins in cereals by LC-FL or LC-MS, where a range of analogues are known, but quantitative standards are only available for fumonisins B1 and B2.

The lack of credible alternative analytical methods for the analysis of marine biotoxins is a strong argument for use of instrumental methods with RRFs as a transitional measure. The poor performance of the traditional MBA is becoming well documented, and most of the proposed in vitro methods have not yet produced precision or accuracy of analytical quality. ELISAs with excellent precision characteristics are becoming available for some toxin groups, but the issues involved in establishing accurate cross-reactivities for analogues are very similar to those for the calibration of instrumental analytical methods.

Conclusions

RRFs are a logical approach in a system of continuous improvement for instrumental methods, and have been used successfully for the OA group. Multi-toxin method validations can be accomplished with a limited range of standards and contaminated tissues. As a wider range of quantitative standards become available: a) limited quantities could be used to establish better RRFs to the primary toxin; and b) larger quantities could be fully incorporated into methods as routine calibrants. Full re-validation of the methods should not be needed.

Reference materials

The available materials, and the need for certified reference materials, have been outlined in the section on Reference Standards and Tissue Materials. In the following section, which materials are needed for method validation and for routine use after validation is specifically stated.

STX group

Although a tissue reference material has already been certified in the EU, the International Weapons Convention, which regulates the distribution of STX as a warfare agent, prevents the distribution of this material. Further efforts should be undertaken by the IRMM to tackle this issue, and to produce more reference materials, including more of the relevant analogues.

Lipophilic toxin groups

The only existing certified standards for the lipophilic group are OA and PTX-2, both supplied by NRC Canada. Therefore, for the application of fully collaboratively validated methods and for the establishment of the relative response factor concept, standards are required for DTX-1 and DTX-2, AZA-1, AZA-2 and AZA-3, PTX-1, YTX, OH-YTX, homo-YTX, and hydroxy-homo-YTX. Once the methods have been validated and the RRF concept has been established, only one standard will be required per group for the routine use of non-animal based methods, either based on LC-MS or based on a functional or biochemical method. The release of certified standards for AZA-1 and YTX in
the near future will promote the further development and validation of replacement methods.

**Interlaboratory studies required**

Interlaboratory studies have been conducted for the LC-FL method for STX developed by Lawrence et al. (93), and for the LC-UV method for DA of Quilliam et al. (68). The method of Lawrence et al. (93) was approved as an official method by the AOAC during 2005 (94, 95). The method of Quilliam et al. (68) has been involved in collaborative trials by two groups (Germany and the EC), and the combined protocol is now undergoing CEN standardisation. All the other methods require further full collaborative trials for validation.

**Summary of research priorities**

The applicability of the TEF, MC and RRF concepts have been demonstrated in principle, and should therefore be validated by using a review of existing data by DG SANCO, in collaboration with the CRL/NRLs, CEN, ECVAM and EFSA.

The priorities for research and development in the area of reference standards and tissue materials, toxicology and method validation, are summarised in Table 3. This clearly shows that a lot of efforts have been completed for the establishment of TEFs and for the in-house validation of methods. However, neither reference standards nor tissue materials are available for most toxins, so the full collaborative studies have not been conducted for most lipophilic toxins. This situation calls for the establishment of an EC programme focused on the production of standards and tissue reference materials.

**Recommendation**

The participants of the workshop recommend that such a programme should be established at the IRMM, and that collaboration between the IRMM and the existing reference materials producer, NRC Canada, should be encouraged.

**The Regulatory Acceptance of Methods in the EU**

Acceptance of non-animal based screening methods in official control

According to Chapter VI of the Annex to Council Directive 91/492/EEC (6), the EU CAs are responsible for establishing methods for the monitoring of bivalve mollusc production areas. The participants of the workshop recommend that the CAs should take into account Directive 86/609/EEC (10) and its requirement that animal testing should be reduced, refined and replaced, and strongly encourage the use of alternative methods, such as the antibody-based LF-IC test kit, LC-FL method or a receptor-based assay for PSP testing, provided that there is sufficient evidence to ensure the required level of consumer safety.

**Table 3: Research priorities in the area of marine biotoxins**

<table>
<thead>
<tr>
<th></th>
<th>Certified materials</th>
<th>Validation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard</td>
<td>Tissue</td>
</tr>
<tr>
<td>OA</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>DTX-1</td>
<td>r</td>
<td>E</td>
</tr>
<tr>
<td>DTX-2</td>
<td>r</td>
<td>R</td>
</tr>
<tr>
<td>DTX-3</td>
<td>r</td>
<td>R</td>
</tr>
<tr>
<td>AZA-1</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>AZA-2</td>
<td>r</td>
<td>R</td>
</tr>
<tr>
<td>AZA-3</td>
<td>r</td>
<td>R</td>
</tr>
<tr>
<td>PTX-1</td>
<td>r</td>
<td>R</td>
</tr>
<tr>
<td>PTX-2</td>
<td>E</td>
<td>R</td>
</tr>
<tr>
<td>YTX</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>OH-YTX</td>
<td>r</td>
<td>R</td>
</tr>
<tr>
<td>Homo-YTX</td>
<td>r</td>
<td>R</td>
</tr>
<tr>
<td>OH-homo-YTX</td>
<td>r</td>
<td>R</td>
</tr>
</tbody>
</table>

*E = exists; R = required with high priority; r = required with lower priority.*
Acceptance of non-animal based reference methods

Commission Decision 2002/225/EC (8) states that functional and biochemical methods, as well as analytical techniques, can be used for all lipophilic marine biotoxins, as an alternative to animal tests, provided that they have been validated in compliance with an internationally accepted procedure. However, it is also stated that, if there are discrepancies in the results obtained with different methods, the MBA should be considered as the reference method. Unfortunately, there is no explanation as to how a non-animal based method can become a reference method, i.e. which criteria should be fulfilled. In the light of the drawbacks of the in vivo methods, it is recommended that the validation of alternative methods as reference methods should only require the demonstration of the same purpose as the bioassay, in that the alternative methods should be adequate for detecting the relevant toxins at the concentrations specified in the legislation.

The roles of the CRL and NRLs

As stated in Council Directive 93/393/EEC (15), one important role of the CRL is to provide scientific advice to the EC. The CRL regularly meets with the NRLs, and at these meetings decisions may be taken with regard to the suitability of methods and whether they should be forwarded to the EC and its Standing Committee for Food Chain and Animal Health.

The role of DG SANCO

The EC and its relevant service, DG SANCO, ensure that shellfish placed on the market are safe for the consumer. On the other hand, it is also essential that other Council Directives, in this case Council Directive 86/609/EEC (10), are respected, i.e. that measures are in place to reduce, refine and replace the animal tests used for marine biotoxin control. DG SANCO can seek the scientific advice of the CRL/NRL network, its Standing Committee for Food Chain and Animal Health and/or the European Food Safety Authority (EFSA) on existing and new methods. Based on the scientific advice provided, the EC can decide to introduce a procedure as a reference method.

The role of the European Food Safety Authority

The mission of EFSA, which began its activities in 2003, is to provide scientific advice and technical support for EU legislation and policies in all fields which have a direct or indirect impact on food and feed safety, and this includes animal health and animal welfare. Through its own scientific expertise and the work of its Scientific Committee and eight Scientific Panels, including the Scientific Panel on Animal Health and Welfare (AHAW), EFSA provides risk assessments on all matters linked with its mission.

The EC can formally address EFSA and ask for the Authority’s scientific opinion on the suitability of methods for marine biotoxin testing. EFSA would then convene an expert panel.

Recommendations

In order to speed up regulatory acceptance, it would be useful to establish criteria to be met by possible non-animal based reference methods. They could be developed by the CRL/NRL network, ECVAM, DG SANCO, EFSA, and organisations as CEN and AOAC, taking into consideration also reduction and refinement procedures for the current in vivo tests or testing strategies.

Strategies for the Implementation of the Three Rs Concept

Immediate possibilities

Until very recently, the regulation of marine biotoxins in shellfish was mainly driven by animal-based testing methods. Therefore, the concept of distinguishing between screening methods and reference methods was underdeveloped in this area. With the current movement toward replacing the animal-based testing with alternative functional and biochemical methods, as well as analytical methods, and insight into the inadequacy of the biological testing methods, it has become evident that the Official Control of marine biotoxins must make use of both screening and reference methods. The use of screening methods in such Official Control is of particular importance for the shellfish commodity, due to the perishable nature of shellfish and the requirement for speed in the Official Control of marine biotoxins.

The concept developed in the area of veterinary residues (84) can easily be applied to the distinction of these two types of approaches for marine biotoxins, and the requirements the methods need to fulfil. Clearly, a qualitative screening method will be sufficient to ensure the safe production of shellfish at the level of monitoring of the product in the water. Some non-animal based methods already fulfil the criteria for qualitative screening methods; for example, the Jellett Rapid Test for PSP (STX group) toxins (73), and the LC-MS methods for the OA, AZA, YTX and PTX groups of toxins (85, 138).
However, to also ensure that processed and imported shellfish have an adequate level of safety, reference methods must be applied, and these should be quantitative and confirmatory in character. Non-animal based methods that already fulfil the criteria for quantitative confirmatory methods include: LC-MS methods for the OA, AZA, YTX and PTX groups of toxins, as far as standards are available (85, 139, 141, 142); the PP2A assay for the OA group toxins (50); the officially-validated LC-FL (pre-column derivatisation) method for STXs (93–95); and modifications to the Oshima LC-FL (post-column derivatisation) method for STXs (37, 142).

The responsibilities and roles of stakeholders in the acceptance of these and other methods for screening and reference methods for official control are described above.

Council Directive 91/492/EEC (6) indirectly allows for this distinction between screening and reference methods in its Annex, Chapter VI, by placing emphasis on the CA to approve methods for the monitoring of live bivalve molluscs. The new hygiene package also clarifies the mandate (and thus the responsibility) of the CAs of the MSs to examine marine biotoxins in live bivalve molluscs. This mandate could be widened to expressly include the recommendation of appropriate methods for the surveillance of production areas and the management of harvesting restrictions.

Medium-term strategies

Following the discussions at the workshop, the participants recommend that the CRL continues to collate, with the support of the NRLs, the validation data available for the non-animal based tests for the determination of PSP toxins, with a view of submitting these data to DG SANCO for their consideration in accordance with Chapter V of the Annex to Directive 91/492/EEC (procedure of article 12 (6)). It is also recommended that the CRL and DG SANCO should organise a workshop to evaluate the TEF, MC and RRF concepts for use in the Official Control of marine biotoxins.

Long-term strategies

The first priority in relation to long-term strategies should be the pursuit of the research requirements outlined in the section on Research Requirements for the Validation of Non-Animal Based Methods, above. Some of the requirements will be addressed in the four current FP6 projects, namely, BIOTOX (58), DetecTox (61), BioCop (82) and BIOTOXMarin (103). However, the major bottleneck is the lack of sustainable production at EU level of certified standards and reference materials for marine biotoxins. This needs to be addressed by DG SANCO and DG Research. DG SANCO should review the adequacy of the current in vivo methods as reference methods — in view of recent toxicological studies and the report produced by the WHO/FAO/IOC expert group (2), and in consultation with EFSA. If appropriate, the current legislation should be amended accordingly.

Summary of Conclusions and Recommendations

Currently-stipulated in vivo methods

1. The participants in the workshop recognise that recent studies demonstrate that the DSP MBA is not an appropriate test for all of the toxin groups covered in Commission Decision 2002/225/EC (8), i.e. OA, DTX, YTX, PTX, and AZA. However, the available non-animal based methods are not fully validated for all of these groups, so the complete replacement of the DSP MBA as a reference method is not yet possible. Nevertheless, the non-animal based methods that have been validated according to internationally accepted protocols should be used for screening in routine monitoring.

<table>
<thead>
<tr>
<th></th>
<th>Detection capability</th>
<th>Detection limit</th>
<th>Trueness/recuperation</th>
<th>Precision</th>
<th>Selectivity/specifity</th>
<th>Applicability/ruggedness/stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qualitative methods</td>
<td>S</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Quantitative methods</td>
<td>S</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

S = screening; C = control.
Moreover, some of the participants felt that this should also apply to single-laboratory validated methods.

2. Despite the fact that the DSP MBA has not been validated and the lack of harmonisation in the protocols used in the EU, the participants in the workshop felt that the DSP MBA should not be officially validated for a number of scientific reasons and animal welfare concerns. The scientific reasons are based on the poor extraction efficiency of the DSP MBA as currently proposed, and its inadequacy to implement the limit for YTXs and an appropriate limit for AZA. The animal welfare reasons include the general pain and suffering to which the test animals are exposed, the lack of harmonisation with regard to length of observation time, the lack of flexibility in the weights of the animals used, and the number of animals per test. DG SANCO has recognised this, and has asked the CRL to work with the NRLs to achieve the EU-wide harmonisation of the DSP MBA. However, this effort should not delay the validation of replacement methods.

3. This opinion is supported by the re-evaluation of limits by the WHO/FAO/IOC expert group (2) called together by the Codex Alimentarius, which suggests that the DSP MBA may be an inappropriate method for achieving the scientific objective of protecting public health from exposure to marine biotoxins at appropriate levels.

4. The CRL and NRLs should evaluate how the described methods for reduction and refinement could be incorporated into the current PSP MBA and DSP MBA protocols. However, this effort should not delay the validation of replacement methods.

Reference materials

5. The EC and the EU MSs are very strongly encouraged to ensure the sustainable provision of pure compounds and tissue reference materials, in order to permit the full validation, regulatory acceptance and implementation of non-animal based methods as reference methods.

6. The participants in the workshop recommend that the EC and the EU MSs should launch an EU programme on the production of standards and tissue reference materials, which could, for example, be established at the IRMM, and should encourage collaboration between the IRMM and the existing reference materials producer, NRC Canada.

TEFs, MCs and RRFs

7. Due to the urgency of the replacement of the animal-based methods in this area, the TEF concept should be agreed upon, and the most appropriate factors and mathematical treatment of data to be used should be determined. For this purpose, DG SANCO should convene a meeting involving international experts and stakeholders.

8. The MC and RRF concepts are important in the Official Control of harvesting areas. The applicability of these concepts has been demonstrated, in principle, and should be fully validated for potential use in reducing animal testing as part of the implementation of non-animal based methods by the MSs.

Validation

9. The reference method for any group of marine biotoxins should be based on performance criteria, thereby allowing the use of several methods, rather than a single specific method. The participants urge WG5 of CEN Technical Committee 275 and the CRL and NRLs to collaborate in the establishment of these criteria. Specifically, the validation of the non-animal based methods should not be based on comparison with the MBA.

10. Further developments (STDs and CRMs) and interlaboratory studies are required to validate non-animal based methods to a level that will be sufficient to justify their use as reference methods.

11. Due to its unique experience in the validation of methods to replace animal tests in the regulatory framework, ECVAM’s advice should be sought for the validation of methods to replace the in vivo methods for marine biotoxin testing.

Acceptance of methods for monitoring and Official Control purposes

12. Since non-animal methods providing equivalent or better public health protection are available, these can serve as monitoring methods, which should be used by the CAs, thereby leading to a significant reduction in animal tests.

13. Taking into account Directive 86/609/EEC (10), it is the responsibility of the CAs of MSs to minimise the use of mice in the surveillance of shellfish production areas (chapter VI of
Directive 91/492/EEC [6]) for PSP toxins through the use of non-animal based methods, such as the Rapid Test (73), LC-FL (93–95) or a receptor based assay (81), provided that there is sufficient data to provide the required level of consumer safety.

14. As a consequence of the workshop, the participants recommend that the CRL should continue to collate, with the support of the NRLs, the validation data available for the non-animal based tests for the determination of PSP toxins, with a view of submitting these data to DG SANCO for their consideration in accordance with chapter V of Directive 91/492/EEC (procedural article 12 [6]).

15. Since new toxicological information has become available since the DG SANCO workshop took place in May 2001, the legislation should be re-evaluated as laid down in Commission Decision 2002/225/EC (8), to reflect the results of recent studies on the toxicity of PTX and YTX, as well as previous risk assessments with regard to AZA.

16. In order to speed up regulatory acceptance, it would be useful to establish criteria to be met by possible non-animal based reference methods. These criteria could be developed by the CRL/NRL network, ECVAM, DG SANCO, EFSA, and organisations such as CEN and AOAC, also taking into consideration reduction and refinement methods relevant to the current in vivo tests or testing strategies.

References

17. Food and Veterinary Office (FVO). Inspection


