CODEX ALIMENTARIUS COMMISSION E

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Agenda Item 3 **CX/FFP 14/33/5**

JOINT FAO/WHO FOOD STANDARDS PROGRAMME

CODEX COMMITTEE ON FISH AND FISHERY PRODUCTS

Thirty-third Session

Bergen, Norway

17 – 21 February 2014

DRAFT PERFORMANCE CRITERIA FOR REFERENCE AND CONFIRMATORY METHODS FOR MARINE BIOTOXINS (SECTION I-8.6 DETERMINATION OF BIOTOXINS) IN THE STANDARD FOR LIVE AND RAW BIVALVE MOLLUSCS
(At Step 6 of the Procedure)

Comments submitted by Australia, Norway and New Zealand

AUSTRALIA

Australia would like to thank the Codex Committee on Methods of Analysis and Sampling (CCMAS) for their consideration of the Draft Performance Criteria and the comments provided. Of particular note to the discussion is:

1. The opinion arising from CCMAS that Mouse Bioassay is a Type 1 method.
2. The recognition of CCMAS that the method criteria needs to be adapted for multi-analyte methods.
3. The decision by CCMAS that toxin equivalency factors (TEF's) need to be included in Appendix VII.
4. The recognition from CCMAS that not all toxin analogs for paralytic shellfish toxins (PST) are listed.
5. The request from CCMAS that a description is included on how total toxicity will be calculated.

Mouse Bioassay as a Type 1 Method

CCMAS has clarified that mouse bioassay is a Type 1 method (REP 13/MAS *Paragraph 23)*. After discussions with the Australian CCMAS representative we accept this clarification and understand that classification as a type 1 method as being related to aspects of the method that are related to the method per se, and therefore cannot be replicated by analytical chemistry. In other words, the mouse bioassay describes the only method for establishing the *level of marine biotoxins in mouse units*, not the only method of establishing marine biotoxins.

Australia understands that this clarification means that the mouse bioassay should no longer be a consideration of this working group, as page 64 of the CAC Procedural Manual (20th ed) states that method criteria approach is relevant to Codex Type II and Type III methods.

We note that, while the mouse bioassay may, in the future, be replaced as a confirmatory method, countries that choose to do so will still be able to use the mouse bioassay as a regulatory tool.

Recognition of CCMAS that the method criteria needs to be adapted for multi-analyte methods

CCMAS noted that "The criteria in the procedural manual were applicable only for single analytes" – this has presented the Codex Committee on Fish and Fishery Products (CCFFP) many issues whilst developing the performance criteria. However, whilst Australia welcomes that CCMAS has stated in Paragraphs 25 and 47 that it will consider development of criteria for the use in determining total toxicity for multi-analyte methods from a general point of view, we consider that waiting for the outcome of this discussion will result

in a considerable delay in the development of the Performance Criteria. Accordingly, Australia would prefer to see work on the Performance Criteria continue in parallel with the CCMAS discussion.

The inclusion of toxin equivalency factors (TEF's) in Appendix VII.

CCMAS has requested information on TEF's for all biotoxin analogs listed (REP 13/MAS Paragraph 26). However, Australia remains of the opinion that the rapid development of science in this area necessitates a different approach.

The reliability and validity of the existing knowledge base on saxitoxin TEFs is the subject of much discussion, and is probably the weakest link in the current capability in determining the safety of shellfish by chromatographic identification and quantification of known congeners. A recent paper by Munday and Reeve (2013) gives an overview of the current state of play, and discusses the assumptions that have been employed in the past, but are becoming increasingly questionable as more work is conducted in this area. Essentially, it is inaccurate to make predictions on oral toxicity from intraperitoneal investigations. Recently acute oral toxicities for some saxitoxin analogs have been published Munday et al (2013), with further work continuing in this area.

Further discussion of the inherent challenges in adopting current TEF-based approaches to seafood safety testing by chemical methods are discussed by Botana et al (2010), and the EFSA expert panel opinion on saxitoxin-group compounds (2009).

A more precise understanding of the relative toxicity of various saxitoxin analogues is developing. Initially this is being informed by suitable animal models using appropriate toxicity end-points and effective sample sizes. This understanding will be to the mutual benefit of both public safety and the seafood industry; however this point will take some time to achieve.

Given the current developments in this field, the previous suggestion of CCFFP that FAO host this information on a webpage that can be more easily updated remains Australia's preferred approach. Australia suggests a note is added to REP 13/FFP Appendix VII to allow incorporation of this information into the document if and when this issue is scientifically resolved. Proposed wording is included in the specific comments below.

PST toxin analogs

CCMAS noted that the AOAC method 2005.06 covered only 12 of the 16 saxitoxin analogues listed in Table 2 (REP 13/MAS Paragraph 21). Australia notes that there are in fact significantly more than 16 PST's; the European Food Safety Authority (EFSA) Scientific Opinion on Marine biotoxins states there are over 30, whilst Weise et al stated in 2010 there are 57 analogs. Lawrence et al (2011) suggests that analogs present at less than 5% of the total amount of toxin should be considered insignificant.

Australia notes that Table 2 in REP 13/FFP Appendix VII is entitled "Toxin analogues to *consider*", and includes a list of toxins that are commonly found. Australia understands that this list is not supposed to be comprehensive. Rather, each competent authority should be assessing the STX congeners in their area, and ensuring they are being appropriately assessed, as detailed by the comments in the method. To clarify this, Australia proposes that additional wording is added to guide appropriate actions if other congeners are identified during analysis (see specific comments below).

The calculation of total toxicity from individual analogs

CCMAS has requested CCFFP provides information on TEF's and how the LOD and LOQ criteria should be applied to the most toxic of the toxins in the STX group (REP 13/MAS Paragraph 22). Australia suggests that, although the maximum regulatory level relates to the total toxicity found in the flesh, the performance criteria should only relate to the analyte being tested, not to the total toxicity. The Procedural Manual allows the development of criteria from a specified method (as the Norwegians have done) and allows determination of criteria for LOD, LOQ, precision and recovery levels for each separate analyte. Total toxicity is then determined by applying the relevant TEF to each analog, and summing the total.

CCMAS also notes difficulties in obtaining reference materials for some analogues. Australia notes that each country must consider this issue according to the toxin profile detected in their monitoring program. Additional wording is suggested below to address this issue.

Way forward

Option 1

Australia would also like to acknowledge the work conducted by the Norwegians and considers that their proposal goes a long way towards a potential resolution. However, Australia notes that the issue with respect to applying a total toxicity to a multi-analogue method remains in the Norwegian table. Accordingly, Australia suggests a solution may be found by following the Norwegian approach, but removing the total toxicity measure from the performance criteria completely: i.e. the performance criteria should only relate to the analyte being tested, not to the total toxicity. Australia notes that it is still necessary to ensure that the performance criteria can meet the maximum level; however, once this has been determined, total toxicity performance criteria do not need to appear in the table.

Australia therefore suggests that Table 1 in the draft Performance Criteria document be replaced with a similar table to that produced by Norway, but with all references to total toxicity removed (see specific comments below).

Australia considers that the comments submitted by Norway have shown that the performance criteria listed for the minimum acceptable level do result in a method that is acceptable for determining the maximum level of total toxicity; however, further information is required to determine if the Limit of Detection (LOD) and Limit of Quantification (LOQ) can do the same. Specifically, Australia recommends that the LOD, LOQ, Precision and Recovery of all saxitoxin (STX) analogs should be given.

Option 2

If agreement cannot be reached between countries on advancing this standard via the current method, it may be necessary to try another approach. It is clear that the current argument centers around the measurement of PSTs; in particular around TEFs (which is a rapidly developing field and which will be resolved by science in due course), and the use of mouse bioassay as a confirmatory test (which countries may never reach agreement on).

As the methods for the okadaic acid group, domoic acid, and azaspiracid group are not in debate, CCFFP should consider listing these methods as reference methods, and leaving the PST method as an unresolved method until the science catches up, and fully validated TEFs are available.

If this approach is taken, it would be necessary to include a paragraph in the bivalve standard that addresses the use of other methods as appropriate regulatory tools in any biotoxin management program. For example, *"In addition to the methods listed, the competent authority may authorize the use of appropriately validated methods, either screening or confirmatory in nature, as appropriate tools for biotoxin management."*

Specific Comments

I-8.6 Determination of Biotoxins

Type II and Type III methods shall be selected in accordance with the "General Criteria for the Selection of Methods of Analysis" and "General Criteria for the Selection of Single-Laboratory Validated Methods of Analysis" in the *Codex Procedural Manual.*

The method selected should be chosen on the basis of practicability and preference should be given to methods which have applicability for routine use.

Methods shall meet the numerical criteria listed in Table 1 and may either meet the minimum applicable range, or LOD and LOQ criteria listed.

~~Multi-analogue method total toxicity criteria are estimated for toxin profiles encountered using validation study data.~~

Rationale: this information is included in the second paragraph under the table.

I-8.6.1 Numerical Criteria Values for Biotoxins in Bivalve Molluscs
Table 1

Toxin Group	Toxin	Minimum applicable range (mg/kg)	LOD (mg/kg)	LOQ (mg/kg)	Precision (RSD_R)	Recovery percent
STX Group	Saxitoxin (STX)	0.05	<insert value>	<insert value>	<insert value>	<insert value>
	(NEO)	0.05	<insert value>	<insert value>	<insert value>	<insert value>
	(dcSTX)	0.05	<insert value>	<insert value>	<insert value>	<insert value>
	GTX1	0.05	<insert value>	<insert value>	<insert value>	<insert value>
	GTX2	0.10	<insert value>	<insert value>	<insert value>	<insert value>
	GTX3	0.10	<insert value>	<insert value>	<insert value>	<insert value>
	GTX4	0.05	<insert value>	<insert value>	<insert value>	<insert value>
	GTX5	0.10	<insert value>	<insert value>	<insert value>	<insert value>
	GTX6	0.10	<insert value>	<insert value>	<insert value>	<insert value>
	dcGTX2	0.10	<insert value>	<insert value>	<insert value>	<insert value>
	dcGTX3	0.10	<insert value>	<insert value>	<insert value>	<insert value>
	C1	0.10	<insert value>	<insert value>	<insert value>	<insert value>
	C2	0.10	<insert value>	<insert value>	<insert value>	<insert value>
	C3	0.5	<insert value>	<insert value>	<insert value>	<insert value>
	C4	0.5	<insert value>	<insert value>	<insert value>	<insert value>
OA Group	OA	0.05	0.01	0.02	<=44%	60-115%
	DTX1	0.05	0.01	0.02	<=44%	60-115%
	DTX2	0.05	0.01	0.02	<=44%	60-115%
Domoic Acid	DA	13.9	1.4	2.8	<=20%	85-110%
AZA Group	AZA1	0.05	0.01	0.02	<=44%	60-115%
	AZA2	0.05	0.01	0.02	<=44%	60-115%
	AZA3	0.05	0.01	0.02	<=44%	60-115%

Total toxicity is estimated as the sum of the molar concentrations of detected analogs multiplied by the relevant specific toxicity equivalency factors (TEFs). Internationally scientifically validated TEFs must be used ~~to calculate total toxicity for methods that do not measure total toxicity directly~~. **The science behind TEFs is rapidly developing. Current internationally validated TEF's can be found on the FAO website. Information on TEFs will be incorporated in this standard at a future date.**

Rationale; CCMAS has requested that information on how to estimate total toxicity is included. There has been considerable discussion at CCFFP around the issue of TEFs, and despite CCMAS advice, Australia

considers this area will continue to change rapidly and should not be prescribed in this standard. See response to CCMAS comments above.

Methods that do not measure total toxicity directly should be validated and used for the relevant toxin analogues that may contribute to total toxicity. Currently known toxin analogues to consider are listed in Table 1.

Where toxin analogues that are not listed in Table 1 are detected above 5% of the total molar toxin content, the competent authority must assess the contribution of these analogs to total toxicity using either known TEFs, or by taking a precautionary approach to assign an interim TEF whilst conducting further investigations.

The competent authority must address the issue of appropriate reference materials for all analogs of concern in the local toxin profiles.

Rationale: to address the CCMAS comments and provide guidance on the saxitoxin analogues not listed in Table 2, and the use of appropriate reference materials. Lawrence et al (2011) suggests that analogs present at less than 5% of the total amount of toxin should be considered insignificant, compounds above this level should be investigated.

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NORWAY

The 32 nd Session of CCFFP advanced a Proposed Draft Performance Criteria for Reference and Confirmatory Methods for Marine Biotoxins in the *Standard for Live and Raw Bivalve Molluscs* to CAC for adoption at step 5 and to CCMAS for endorsement.

The 36 th Session of CAC adopted the draft at step 5 and advanced it to step 6 for consideration by the 33 rd Session of CCFFP. The 34 th Session of CCMAS did not endorse the method criteria for biotoxins, and encouraged CCFFP to provide information on the toxicity equivalent factors for all biotoxins listed in the standard, and applying criteria for LOD and LOQ for the most toxic of the toxins in the Saxitoxin group. Some CCMAS delegations expressed the view that the criteria specified in the Procedural Manual were adequate for chemical methods but were not applicable for biological methods such as mouse bioassay. It was clarified that the mouse bioassay had been proposed for biotoxins as Type I method and therefore criteria were not applicable. It was also noted that it is not possible to endorse both Type I and Type II methods for the same provision. CCMAS agreed that the development of criteria in case of total toxicity should be considered from a more general point of view and will consider development of criteria for multi component methods for their next session 2014.

Analytical methods for determination of biotoxins are evolving and hopefully several chemical analytical methods will be made available, which in the future will be replacing biological assays. Having method performance criteria would allow flexibility in the choice of which method to apply. The current method criteria in Codex are based and described for chemical methods for determination of single components. The Procedural Manual also describes converting recommended methods into criteria.

For biotoxins, the total toxicity is estimated as the sum of the concentrations of individual components (analogues) of toxin groups multiplied with specific toxicity equivalent factors (TEFs). In order to determine the total toxicity, any appropriate chemical analytical method has to be able to determine the analogues with satisfactory performance. As the requirements for the performance of the analogues would be the same as for other single components, the method performance criteria described in the Procedural Manual could very well be applied. The determinations of the analogues are independent of the TEFs, as the concentrations of the components are determined separately, and hence the methods are not in the category of being Type I methods.

Method Criteria and TEFs

In the Working Instruction for the Implementation of the Criteria Approach in Codex (Procedural Manual p. 65) it is stated that in some cases the Committee may find it easier to recommend a specific method and "convert" that method into appropriate criteria. In this document it is proposed to convert specific methods for analyses of biotoxins into method criteria. The recommended methods for conversion into criteria are interlaboratory validated methods from AOAC International, NMKL (Nordic Committee on Food Analysis), the European Committee for Standardization and from the European Union Reference Laboratory for Marine Biotoxins. The results from the interlaboratory studies of the methods are reviewed in particular with respect to the lowest validated levels with satisfactory precision.

Generally, the TEFs have higher uncertainty than the analytical measurements (for further reading, see references 1-4). It is acknowledge that TEFs are reviewed as new toxicological data becomes available. TEF values for the saxitoxin group are given in Annex 1. For the purpose of setting method criteria, and taking the uncertainty of the TEF values into account, the suggested TEF values for this purpose are given in a separate column using only one significant digit.

All methods need to be able to detect and determine the analogues in a particular toxin group to a sensitivity that protects the consumer. Because of this, the TEFs should influence the limit of quantification (LOQ) for the toxin analogues in the method. For instance, the sensitivity should be about 10-fold lower for analogues that are about ten-fold more toxic compared to others. This has been considered in Annex 2. In Annex 2, the lowest validated levels with satisfactory precision for the analogues of the toxin groups obtained in interlaboratory studies of the specific methods are also given. The results of the studies together with the calculated LOQs based on TEFs form the basis of the proposed criteria (Annex 2). The proposed criteria along with the methods fulfilling the criteria are given in Table 1 of this document.

The maximum level, ML, is given for the total toxicity of the toxin group of interest, which consist of several analogues. As the current Codex method criterion for the precision is based on Horwitz/Thompson equation, which is valid for single chemical components only, this cannot be applied to multi component methods. This is briefly illustrated in Annex 3; "Why Horwitz/Thompson equation is not valid for multi component methods".

Methods, where the individual compounds are not determined, e.g. bioassays/ mouse tests, ought to be validated against any of the methods fulfilling the method performance criteria. If it can be demonstrated through an interlaboratory study (method performance study or proficiency testing schemes) that the method of interest provides equivalent results, the method could be considered appropriate.

Conclusions

Norway recommends that the 33rd Session of CCFFP considers the following:

- the toxicity equivalent factors (TEFs) given in Annex 1 (Norway's proposal is the column For Codex purposes to the far right) for PSP and in Table 1 of this document for the OA and the AZP groups.
- the method criteria suggested in Table 1 of this document based on the considerations made in Annex 2. To recommend numeric values for the method performance (LOD, LOQ, minimum applicable level (mAL), and precision (RSD/HorRat)) of the analogues for the toxin groups based on the TEFs.

Notifying that the current Codex criterion for precision is not applicable for the sum of the toxins (according to Annex 3).

- to include and review other appropriate methods fulfilling the criteria, and further compare results obtained by bioassays against chemical methods. Norway requests PT-scheme providers to forward results from PT-schemes were different methods for analyses of biotoxins have been used. If the reviewed results are satisfactory, bioassay methods could be agreed upon at the session and included as method suggested as appropriate.

Table 1: Method performance criteria for biotoxins in bivalve molluscs

Commodity	Provision	Maximum level, ML	Method performance criteria	Appropriate Methods
Bivalve Molluscs	Total Toxicity of Saxitoxin (STX) group See Annex 1for the analogues and its TEF	0.8 mg/kg STX·diHCl eq	Criteria based on AOAC 2011.02 (see Annex 2 A) For the individual analogues, with TEF ≥ 1 Minimum applicable level ≤ 0.05 mg/kg LOD ≤ 0.01 mg/kg LOQ ≤ 0.02 mg/kg Precision: RSD ≤ 44% 0.1< TEF < 1 Minimum applicable level ≤ 0.1 mg/kg LOD ≤ 0.03 mg/kg LOQ ≤ 0.05 mg/kg Precision: HorRat ≤ 2 TEF ≤ 0.1 Minimum applicable level: ≤ 0.5 mg/kg LOD ≤ 0.1 mg/kg LOQ ≤ 0.2 mg/kg Precision: HorRat ≤ 2 Recovery: 50 - 130%	AOAC 2011.02 NMKL 197 (2013) HPLC–fluorescence AOAC 2005.06 EN 14526:2004 NMKL 182 (2005) HPLC–fluorescence AOAC 2011.27 Receptor binding assay Mouse test (tested against AOAC 2005.06, See Annex 4) [AOAC 959.08 Bioassay Mouse test - See Annex 4 against AOAC 2005.06 – more data needed?]
Bivalve Molluscs	Total Toxicity of Okadaic acid (OA) group **TEFs of OA group:** OA: 1.0 DTX1: 1.0 DTX2: 0.5	0.16 mg/kg OA eq	Based on the EU SOP[1] (See Annex 2B) For the individual analogues, with TEF ≥ 1 mAL ≤ 0.04 mg/kg LOD ≤ 0.01 mg/kg LOQ ≤ 0.03 mg/kg Precision: RSD ≤ 44% TEF ≤ 0.5 mAL ≤ 0.1 mg/kg LOD ≤ 0.03 mg/kg LOQ ≤ 0.05 mg/kg Precision: HorRat ≤ 2 Recovery: 60 -115%	"EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS[1] EU harmonized standard operation procedure for determination of OA Group toxins by LC-MS/MS[2] EN 16204:2012 LC-MS/MS
Bivalve Molluscs	**Domoic acid**	20 mg/kg	From Procedural Manual Minimum applicable level: 14 mg/kg LOD: 2 mg/kg LOQ: 4 mg/kg Precision: HorRat ≤ 2	EN14176:2003 HPLC EU-RL-MB Standard operating procedure for

Commodity	Provision	Maximum level, ML	Method performance criteria	Appropriate Methods
			Recovery: 85-110% (based on method validation)	determination of domoic acid[3] (ASP toxins in molluscs by UPLC-MS EU harmonized SOP; HPLC UV[4] AOAC 2006.02 ELISA [AOAC 991.26 HPLC 20 mg/kg]
Bivalve Molluscs	**Azaspiracid (AZP) group** **TEFs of AZP group:** AZA-1: 1.0 AZA-2: 1.8 AZA-3: 1.4	0.16 mg/kg AZA eq	Based on the EU SOP[1] (see Annex 2B) For the individual analogues with TEF ≥ 1 Minimum applicable level ≤ 0.03 mg/kg LOD ≤ 0.01 mg/kg LOQ ≤ 0.02 mg/kg Precision: RSD ≤ 44% Recovery: 40 -120%	Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS[1] EN 16204:2012 LC-MS/MS

1) Link to the Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS" http://www.aesan.msssi.gob.es/CRLMB/docs/docs/ayuda_cientifica/report__inter.pdf
2) The method is available at the link: http://www.aesan.msps.es/CRLMB/docs/docs/procedimientos/EU-Harmonised-SOP-LCMS-OA-Version1.pdf
3) The method is available at the link: http://www.aesan.msps.es/CRLMB/docs/docs/metodos_analiticos_de_desarrollo/EURLMB_SOP_Domoic_acid__UPLC-MS.pdf
4) The method is available at the link: http://www.aesan.msps.es/CRLMB/docs/docs/procedimientos/EU-Harmonised-SOP-ASP-HPLC-UV_Version1.pdf

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Annex 1 Toxicity Equivalent Factors (TEFs) for the PSP toxins

Table A1 TEFs for the PSP toxins

PSP TOXIN	TEF Oshima 2004	TEF EFSA 2009	TEF based on MBA Munday *et al.* 2013	Relative LD_{50} by i.p. injection Munday *et al.* 2013	Relative LD_{50} by feeding Munday *et al.* 2013		For Codex purposes
STX	1.000	1.0	1.00	1.00	1.00		1
GTX1	0.994	1.0	--	--	--		1
GTX2	0.359	0.4	--	--	--		0.4
GTX3	0.638	0.6	--	--	--		0.6
GTX4	0.726	0.7	--	--	--		0.7
GTX1,4 (80, 20%)	--	--	1.02	1.90	0.93		1*
GTX2,3 (68, 31%)	--	--	0.60	0.76	0.57		0.6*
GTX5 (B1)	0.064	0.1	--	--	--		0.1
GTX6 (B2)	0.064	0.1	--	--	--		0.1
dcGTX2	0.154	0.2	--	--	--		0.2
dcGTX3	0.377	0.4	--	--	--		0.4
C1 (epi-GTX8)	0.006	--	--	--	--		0.006
C2 (GTX 8)	0.096	0.1	--	--	--		0.1
C3	0.013	--	--	--	--		0.01
C4	0.058	0.1	--	--	--		0.1
NEO	0.924	1.0	1.16	3.12	2.54		1
dcSTX	0.513	1.0	0.64	0.79	0.37		0.6
dcNEO (GTX 7)	--	0.4	--	--	--		0.4
11-hydroxy-STX	0.319	0.3	--	--	--		0.3
B. Ben-Gigirey et al. (publication in press 2014)						sum	7*

(received from Dr. Ana Gago-Martínez , EU Reference Laboratory for Marine Biotoxins)

*The TEFs of GTX1,4 and GTX3,4 are excluded in the sum as the induvidual TEFs for GTX1, GTX2, GTX3 and GTX4 are included.

Annex 2: Criteria based on conversion of methods and calculation of LOQ from TEF

A) Criteria for Paralytic Shellfish Poisoning Toxins, PSP

In establishing criteria, results from the following methods have been used:

- AOAC 2011.02 / NMKL 197: Paralytic Shellfish Toxins in Mussels, Clams, Oysters, and Scallops. Post-Column Oxidation (PCOX) Method

From the interlaboratory studies, the lowest validated levels with satisfactory precision, according to Horwitz /Thompson equation, for the analogues are given in Table A2.1.

It has been suggested to base the criteria on the limit of quantification, LOQ, for each toxin analogue, taking the TEF factor into consideration. When an analogue is 10-fold more toxic than another analogue, the LOQ should be about 10 times lower. And if there are many toxin analogues in a toxin group that should also affect the LOQ (e.g. if there were 10 analogues of TEF=1, the LOQ should be affected by a factor of $1/10^{th}$ to the maximum limit, ML, for ensuring food safety.)

In Codex, the method criteria for LOQ = 1/5·ML for levels above 0.1 mg/kg and 2/5·ML for levels below 0.1 mg/kg. Mathematically, the required LOQ for analogue X in toxin group Y could then be expressed relative to parent analogue Z (the one whose TEF=1);

$$LOQ(Z) = \frac{ML \cdot \frac{1}{5} \cdot 1}{\sum TEF(Y)} \qquad (1)$$

$$LOQ(X) = \frac{LOQ(Z)}{TEF(X)} \qquad (2)$$

For the analogues of the PSP toxin, using the "Codex recommended TEFs", the sum of TEF, ∑TEF(Y) would be 7 eq. (given in Annex A) and ML = 0.8 mg/kg eq. From equation 1 and 2 these give;

LOQ(Z) = 0.8 mg/kg eq · 1/5 · 1 eq / 7eq = 0.02 mg/kg eq

For TEF=1, the LOQ would then be: 0.02 mg/kg eq/1eq = 0.02 mg/kg

According to the Codex criteria, the limit of detection, LOD, is ½ of the LOQ. Numeric values for the minimum applicable level, mAL, for each analogue can easily be obtained by using the Codex criteria which are available at NMKLs webpage www.nmkl.org under "Excel Spreadsheet for downloading", "How to get method criteria based on ML".

The table below shows the results of the interlaboratory study, the criteria based on the calculated LOQ from TEF values, and finally the proposed criteria based on the combination of the two. It shows that the results obtained in interlaboratory study are satisfactory and that the method is able to determine low levels for all analogues. As the analogues with very low TEFs contribute less to the total toxicity compared to analogues with TEF close to 1, the lowest validated levels with satisfactory precision do not need to be that low as obtained in the method validation. Thus, the calculation of the LOQ as a criterion has been considered too. On the other hand, the criteria based on the calculation of the LOQ, might be too loose, as the sum of mAL derived from the LOQ calculations multiplied with the respective TEF factors might exceed the ML. Therefore, a combination of converting the method into criteria and criteria based on calculations of LOQ has been considered. When it comes to recovery, the validations have shown that the criteria given in the Codex Procedural Manual are too strict for these analyte and matrices, and the recovery has been validated to 50-130%.

Table A2.1 The analogues of Saxitoxin group, the lowest validated level with satisfactory precision obtained in the interlaboratory study of AOAC 2011.02/NMKL 197, the TEFs, the calculated criteria based on TEF, and the proposed criteria based on the results of the study and the calculated criteria based on the TEFs

Analogues	**Lowest validated level (mg/kg)**	**TEF**	**Calculated criteria based on TEF* (mg/kg)**			**Proposed criteria based on method validation results and the calculated criteria based on TEF**
			LOD	LOQ	mAL	
STX	0.04	1	0.01	0.02	0.03	TEF ≥ 1 mAL ≤ 0.05 mg/kg LOD ≤ 0.01 mg/kg LOQ ≤ 0.02 mg/kg Precision: RSD ≤ 44%
NEO		1	0.01	0.02	0.03	
GTX 1	0.07	1	0.01	0.02	0.03	
GTX 4	0.06	0.7	0.02	0.03	0.05	0.1< TEF < 1 mAL ≤ 0.1 mg/kg LOD ≤ 0.03 mg/kg LOQ ≤ 0.05 mg/kg Precision: HorRat ≤ 2
dcSTX	0.04	0.6	0.02	0.04	0.08	
GTX 2	0.14	0.4	0.03	0.06	0.1	
GTX 3	0.06	0.6	0.02	0.04	0.08	
dcGTX3	0.04	0.4	0.03	0.06	0.1	
dcGTX2	0.14	0.2	0.06	0.11	0.3	
GTX 5 (B-1)	0.14	0.1	0.11	0.23	0.5	TEF ≤ 0.1 mAL ≤ 0.5 mg/kg LOD ≤ 0.1 mg/kg LOQ ≤ 0.2 mg/kg Precision: HorRat ≤ 2
C-1	0.05	0.006	1.90	3.81	13	
C-2	0.05	0.1	0.11	0.23	0.5	
C-3	-	0.01	1.14	2.29	7.3	
C-4	-	0.1	0.11	0.23	0.5	

***** LOQ(Z)=0.8 mg/kg·1/5·1/7 (0.8 is the ML, 1/5 is due to levels above 0.1 mg/kg, 7 is the sum of TEF (Annex 1)). LOQ(X) =LOQ(Z)/TEF(X), LOD and mAL are found in the NMKL Excel sheet based on the Codex Criteria, corresponding to ML 0.1, 0.15, 0.2, 0.3, 0.55, 1, 11 and 19 mg/kg, respectively.

B) Criteria for lipophilic toxins

Results from the following method have been used:

- EU-RL-MB: Interlaboratory Validation Study of the EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS.

The method has amongst others been validated on OA-group toxins and AZA-group toxins. Table A2.2. shows the results from the validation study, criteria obtained by calculating LOQ from TEF, and finally the proposed criteria based on the combination of the two.

Table A2.2 The analogues of toxin groups, the lowest validated level with satisfactory precision obtained in the EURLM interlaboratory studies, the TEFs, the calculated criteria based on TEF, and the proposed criteria based on the results of the study and the calculated criteria based on the TEFs

OA Analogues	**Lowest validated level (mg/kg)**	**TEF**	**Calculated criteria based on TEF* (mg/kg)**	**Proposed criteria based on method validation results and the calculated criteria based on TEF**
Okadaic acid, OA	0.06	1	By calculation according to equation 1&2: TEF=1: LOQ = 0.03 mg/kg, LOD = 0.01 mg/kg, mAL =0.04 mg/kg TEF=0.5: LOQ = 0.05 mg/kg,	TEF ≥ 1 mAL ≤ 0.04 mg/kg LOD ≤ 0.01 mg/kg LOQ ≤ 0.03 mg/kg Precision: RSD ≤ 44% TEF ≤ 0.5 mAL ≤ 0.1 mg/kg LOD ≤ 0.03 mg/kg
Dinophysistoxin-1 (DTX1)	0.1	1		
Dinophysistoxin-2 (DTX2)	0.04	0.5		
	∑TEF	2.5		

			LOD=0.03 mg/kg; mAL=0.1 mg/kg In the SOP[2](ref.under table 1) the criteria for mAL = 0.04 mg/kg	LOQ ≤ 0.05 mg/kg Precision: HorRat ≤ 2
AZA Analogues				
AZA 1	0.04	1	By calculation according to equation 1&2: TEF=1: LOQ = 0.02 mg/kg LOD = 0.01 mg/kg, mAL = 0.03 mg/kg TEF= 1.8: LOQ = 0.01 mg/kg, LOD = 0.006 mg/kg, mAL = 0.02 mg/kg	TEF ≥ 1 mAL ≤ 0.03 mg/kg LOD ≤ 0.01 mg/kg LOQ ≤ 0.02 mg/kg Precision: RSD ≤ 44%
AZA 2	0.02	1.8		
AZA 3	0.02	1.4		
	∑TEF	4.2		

***** LOQ(Z)=0.8 mg/kg·1/5·1/7 (0.8 is the ML, 2/5 is due to levels below 0.1 mg/kg, 7 is the sum of TEF (Annex 1). LOQ(X) =LOQ(Z)/TEF(X), LOD and mAL are found in the NMKL Excel sheet based on the Codex Criteria.

Annex 3 Why Horwitz/Thompson equation is not valid for multi component methods

The total toxicity is the sum of the concentration of the analogues multiplied with the respective TEF.

At the minimum applicable level, mAL, this is expressed as ∑mAL·TEF

In the Codex Procedural Manual, the criterion for the precision is given as the relative standard deviation. The relative standard deviation, RSD, is expressed as the following:

$$RSD(\%) = \frac{s}{x} \cdot 100\% \Leftrightarrow s = \frac{RSD \cdot x}{100} \qquad (1)$$

where *s* is the standard deviation and *x* is the concentration (here: x= mAL·TEF).

The standard deviation for the total toxicity would be the combined uncertainty of the standard deviation of the analogues, i.e. the sum of the variances of the standard deviation, s^2, of the analogues of interest.

$$s_{total} = \sqrt{\sum s_i^{\,2}} = \sqrt{(\frac{RSD_i}{100} \cdot mAL_i \cdot TEF_i)^2} \qquad (2)$$

The relative standard deviation of the total toxicity, RSD_{total}, at minimum applicable level (mAL), is the square root of the sum of the variances of the individual compounds divided by the concentration:

$$RSD_{total}(\%) = \frac{\sqrt{\sum (\frac{RSD_{Ri}}{100} \cdot mAL_i \cdot TEF_i)^2}}{\sum (mAL_i \cdot TEF_i)} \cdot 100\% = \frac{\sqrt{\sum (RSD_{Ri} \cdot mAL_i \cdot TEF_i)^2}}{\sum (mAL_i \cdot TEF_i)} \qquad (3)$$

Depending on the TEF values, the mALs for PSP are 0.05, 0.1 and 0.5 mg/kg, respectively, (see Annex 2) (corresponding to maximum level of 0.14, 0.25 and 1 mg/kg in the Codex criteria, using the Excel Spreadsheet at the homepage of NMKL). For these levels the RSD will vary from 32 - 44%. The numbers used in the calculations are given in the table below.

Table 3.1 The TEF, mAL and RSD of the PSP toxins, and the combination thereof for the use in the estimation of the relative standard deviation of the total toxicity, RSD_{total}

PSP TOXIN	TEF	mAL (mg/kg)	TEF· mAL (mg/kg eq.)	RSD(%)	$(mAL \cdot TEF \cdot RSD)^2$ $(mg/kg\ eq\ \%)^2$
STX	1	0.05	0.05	44	4.84
GTX1	1	0.05	0.05	44	4.84
GTX2	0.4	0.1	0.04	39	2.43
GTX3	0.6	0.1	0.06	39	5.48
GTX4	0.7	0.1	0.07	39	7.45
GTX5 (B1)	0.1	0.5	0.05	32	2.56
GTX6 (B2)	0.1	0.5	0.05	32	2.56
dcGTX2	0.2	0.1	0.02	39	0.608
dcGTX3	0.4	0.1	0.04	39	2.43
C1 (epi-GTX8)	0.006	0.5	0.003	32	0.0092
C2 (GTX 8)	0.1	0.5	0.05	32	2.56
C3	0.01	0.5	0.005	32	0.026
C4	0.1	0.5	0.05	32	2.56
NEO	1	0.05	0.05	44	4.84
dcSTX	0.6	0.1	0.06	39	5.48
dcNEO (GTX 7)	0.4	0.1	0.04	39	2.43
11-hydroxy-STX	0.3	0.1	0.03	39	1.37
Sum	**7**		**0.718**		**52.48**

Using formula (3) to estimate the relative standard deviation of the total toxicity, the following is obtained:

$$RSD_{total}(\%) = \frac{\sqrt{4.84 + 4.84 + 2.43 + \ldots + 1.37}}{0.72} = 10\%$$

A relative standard deviation for the total toxicity of 10% is very tight. If only the five first analogues of the PSP toxins in table 3.1 were present (STX, GTX1, GTX2, GTX3 and GTX4), the RSD_{total},would be 19%. If only STX was present, the RSD_{total}, would be 44% . And when having only one analogue, this would be according to the numeric value obtained by using the Horwitz/Thompson equation.

The ML for the total toxicity for the PSP is 0.8 mg/kg STX·diHCl eq. For a single component with concentration of 0.8 mg/kg, the predicted RSD_R is 33%. If this should be the requirement for the total toxicity, and all analogues were present, the RSD of each analogue could be above 100%, which is not satisfactory.

The RSD of the total becomes smaller when the number of components increases. The more components, the narrower the RSD become. This can easy be illustrated if assuming that for *n* analogues the TEF=1, mAL=1 and RSD=44%

$$RSD_{total} = \frac{\sqrt{\sum (RSD_{Ri} \cdot mAL_i \cdot TEF_i)^2}}{\sum (mAL_i \cdot TEF_i)} = \frac{\sqrt{\sum (44_1 \cdot 1_1 \cdot 1_1)^2 + (44_2 \cdot 1_2 \cdot 1_2)^2 + \ldots + (44_n \cdot 1 \cdot {}_n 1_n)^2}}{\sum (1_1 \cdot 1_1) + \ldots + (1_n \cdot 1_n)}$$

$$= \frac{\sqrt{44^2 \cdot n}}{n} = \frac{44\sqrt{n}}{n}$$

When n increases, the RSD_{total} becomes smaller.

This shows that Horwitz /Thompson equation cannot be applied to multi component results, nor does it make sense to set criteria for the precision for a sum of components, based on the equation above, as the precision will be smaller though more analogues present.

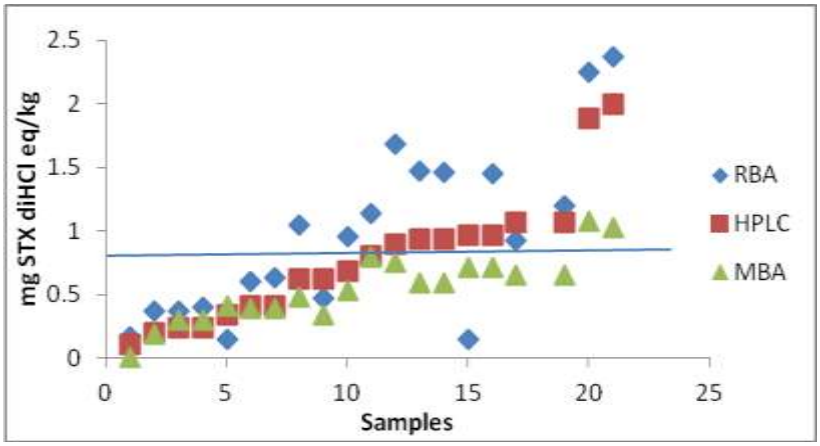
Annex 4 Comparison of methods for paralytic shellfish toxins

Source : Van Dolah, Frances M.; Fire, Spencer E.; Leighfield, Tod A.; Mikulski, Christina M.; Doucette, Gregory J.. Determination of Paralytic Shellfish Toxins in Shellfish by Receptor Binding Assay: Collaborative Study, *J. AOAC Int. 95, 795 (2012)*

During the validation of AOAC 2011.27, a microplate format receptor binding assay (RBA) for paralytic shellfish toxins (PST)

was compared against the HPLC method (AOAC 2005.06) and the mouse bioassay (AOAC 959.08). A total of 21 shellfish homogenates were analysed. Nine laboratories analysed according to AOAC 2011.27, one laboratory analyzed the samples using the precolumn oxidation HPLC method (AOAC 2005.06) to determine the STX congener composition. Three laboratories performed the mouse bioassay (AOAC 959.08). The study focused on the ability of the assay to measure the PST toxicity of samples below, near, or slightly above the regulatory limit. The results are shown in the table and figure below, in the increasing content based on the HPLC method. The bold figures are results at and above the maximum level. It shows that at levels close to and above the maximum level of 0.8 mg/kg STX diHCl eq, the MBA method provides lower results than the HPLC and the RBA method.

Sample	**RBA**	**HPLC**	**MBA**
AOAC	**2011.27**	**2005.06**	**959.08**
1	0,168	0,108	-
2	0,365	0,196	0,182
3	0,371	0,236	0,299
4	0,403	0,236	0,299
5	0,149	0,341	0,405
6	0,599	0,413	0,387
7	0,627	0,413	0,387
8	1,051	0,618	0,485
9	0,466	0,625	0,343
10	**0,96**	0,685	0,528
11	**1,134**	**0,802**	**0,792**
12	**1,683**	**0,894**	**0,752**
13	**1,476**	**0,931**	0,595
14	**1,46**	**0,931**	0,595
15	**0,144**	**0,965**	0,714
16	**1,452**	**0,965**	0,714
17	**0,926**	**1,07**	0,653
19	**1,203**	**1,07**	0,653
20	**2,252**	**1,89**	**1,08**
21	**2,374**	**2**	**1,027**



NEW ZEALAND

New Zealand notes the discussion in CCMAS with respect to marine biotoxin test method criteria and makes the following comments.

The reason for the criteria approach being taken was originally because of the lack of agreement in CCFFP as to the appropriate reference method for each toxin group.

It remains an ideal situation to have reference methods. It would appear that most countries are using HPLC methods for domoic acid detection (LC-MS in New Zealand, which correlates well with the HPLC method). Many are now using LC-MS for the okadaic acid group and azaspiracids rather than the lipophilic toxin mouse bioassays previously used because these give a large number of false positives and some false negative results.

Brevetoxins are reported from the United States and have been a problem once in New Zealand and not been seen for the past 20 years in any significant amount. To our knowledge they are not tested for in products in international trade.

The main area of contention remaining is around the use of mouse bioassays for saxitoxin group detection which is being progressively phased out in many countries versus the chemical methods and functional methods. The chemical methods currently in use are heavily reliant upon mouse bioassay studies of the toxicity of individual toxin analogues by intra-peritoneal injection to establish toxic equivalency factors (TEFs). However, oral toxicity studies are underway for these toxin congeners in more than one country including New Zealand and the results are showing significant discrepancies between the oral toxicity and the Intra-peritoneal toxicity in some cases, some analogues are significantly more toxic orally and in others they are less toxic.

Further, from toxic shellfish poisoning case information in the New Zealand situation, it is apparent that where saxitoxin and neo-saxitoxin are present in shellfish, there is likely insufficient safety margin between the current 0.8 mg/kg and the point where consumers are falling ill relative to safety margins applied for other contaminants in food. Other toxin analogues in our waters (predominantly C toxins) have not to our knowledge resulted in illness even at 20-30mg/kg in shellfish consumed.

New Zealand proposes that in light of the above, an option the Committee should consider to progress the biotoxin methods section of the Bivalve Shellfish Standard could be to:

- Establish reference methods for domoic acid and its isomers, the okadaic acid group and azaspiracids.
- Remove limits and test methods for brevetoxins because these are not actually being tested for in products in international trade.
- Insert “To be developed” against the saxitoxin group reference method pending further scientific advances to establish proper oral toxic equivalency factors. A footnote should be inserted to that effect and noting that there are a variety of methods currently in use world - wide that provide a reasonable level of protection.

We append a copy of a recent New Zealand paper that describes why IP toxicological studies and hence test methods based on them are inappropriate. This paper is submitted as part of our comments and we request the Committee take this information into account in its deliberations on this topic.

In New Zealand’s view, the concept of “total toxicity” based on IP mouse bioassay is erroneous, and creates a two-fold problem. The first being over–estimation of risk from some toxin analogues and the second being under-estimation of risk from some toxin analogues. The continued use of mouse bioassays is preventing the setting of limits with appropriate safety margins as we would for other contaminants. The same problem has occurred with the lipophilic toxins and this has largely been resolved by testing for specific compounds. The same thing needs to occur for the saxitoxin group of compounds.

The criteria agreed at the 2012 CCFFP meeting were the result of much compromise on the part of many delegations to achieve a result. A key component of that compromise was not including TEFs because of the lack of consensus. If the criteria approach is to be continued, New Zealand can only agree to TEFs derived from oral toxicity studies being included. New Zealand does not agree with TEFs from IP studies being included.

Screening versus Reference Methods

There is a secondary, and as yet unresolved discussion about the relationship between validated screening methods and reference methods – some countries insist reference methods are used for testing products in trade. Others are of the view that validated screening or other confirmatory methods than the reference method should be sufficient provided that they are validated to ensure that product that passes these screening methods will always pass the relevant reference method.

New Zealand is firmly of the view that it should not matter what method is used for screening products provided that when tested by a reference method the product tests within acceptable limits. If it can be agreed to insert wording to this effect into the Bivalve Shellfish Standard this may assist in resolving the difficulty countries have with reference methods that they are not currently able to implement due to technical complexity or cost.