



**JOINT FAO/WHO FOOD STANDARDS PROGRAMME**  
**CODEX COMMITTEE ON METHODS OF ANALYSIS AND SAMPLING**  
**38<sup>th</sup> Session**  
**Budapest, Hungary, 8 - 12 May 2017**

**CRITERIA FOR ENDORSEMENT OF BIOLOGICAL METHODS USED  
TO DETECT CHEMICALS OF CONCERN**

(Prepared by the eWG chaired by Chile & France)

**BACKGROUND**

The Codex Committee on Methods of Analysis and Sampling, at its 35th session (March 2014) endorsed the Criteria for determination of toxin analogues by chemical methods in the section 1-8.6.1 of the Standard for live and raw bivalve molluscs, as well as the classification of the methods AOAC 959.08 (mouse bioassay) and AOAC 2011.27 (receptor binding assay) as Type IV, in the section I-8.6.2 of that Standard. (*Reference to foot #1: REP14/MAS, para.23-25*). During the 37th session of the Codex Alimentarius Commission (July 2014), the draft sections I-8.6.1 and I-8.6.2, endorsed and amended by CCMAS, were considered. There were concerns regarding the classification of the mouse bioassay as Type IV, which it was suggested could not be used for control, inspection and regulatory purposes. Some delegations expressed the view that the CCMAS should consider developing criteria for biological methods as the current criteria used for section of methods applied to chemical methods, and led to the Type IV classification. As a result of the debate, the CAC returned section I-8.6.2 to CCMAS with a request to review the typing of the methods in question, and encouraged CCMAS to proceed rapidly with its discussion on the way to deal with biological methods from a criteria approach perspective. (*Reference to foot #2: REP14/CAC, para. 53-60*).

At the 36th CCMAS session (February 2015), the request of the Commission to review the typing of the methods for determination of marine biotoxins was considered. After an extensive discussion on the types of methods used to quantify marine toxins (chemical and biological), the Committee agreed to maintain its endorsement of the methods in section I-8.6.2 as Type IV and agreed that the development of criteria for biological methods should be considered as a matter of urgency as also encouraged by the Commission. The CCMAS established a eWG led by Chile and co-chaired by France, with the following mandate:

- i) classify biological methods according to their nature, principles, characteristics, etc.
- ii) identify to which classes of the method criteria approach applies, and recommend criteria to endorse each class of biological methods identified in step i
- iii) For the purpose of this working group, biological methods are considered to be those methods of analysis which uses whole or parts of organisms as analytical indicators, excluding PCR, enzymatic and ELISA.

Also, the methods used for the assessment of food hygiene were outside the scope of the eWG, which fall within the remit of CCFH. (*Reference to foot #3: REP15/MAS, para. 44-59*).

At the 37th CCMAS session (February 2016), the Delegations of Chile and France presented the *Discussion paper on criteria for endorsement of biological methods used to detect chemical of concern*, and explained that the eWG had only addressed the first point of its mandate (methods classification). (*Reference to foot #4: CX/MAS 16/37/6*).

The eWG noted that most of the biological methods typed in Codex were Type II and III, with one Type I method (rat bioassay for determination of the protein efficiency ratio), while the methods for determination of marine biotoxins were Type IV. In addition, it was noted as an obstacle the lack of revision of the list of methods in CODEX STAN 234-1999, because there are no longer provisions for some of them, which could be removed or considered by the Committee (e.g. methods for minarine and margarine, as well as the current use of chromatographic methods for the determination of vitamins).

During the session a general discussion was held and the proposal to clean up the list of biological methods was consulted in consultation with the relevant committees to identify what kind of methods the criterion would apply and to avoid defining criteria for methods in general. Where some countries argued that biological methods could be replaced by instrument-based methods and therefore biological criteria would not be necessary.

The Committee agreed to re-establish the eWG chaired by Chile and co-chaired by France, working in English to identify those methods already adopted by Codex as possible replacements for some of the biological methods for determination of vitamins, and to identify clear questions that could be asked to the relevant Codex committees in relation to these methods; to continue with the classification of biological methods; and to identify to which classes of methods the criteria approach applies and recommend criteria to endorse each class of biological methods defined. (*Reference to foot #5:REP16/MAS, para. 64-70*).

The eWG was attended by 18 countries and 1 organization (the list of participants is attached as Appendix I).

The EWG has prepared a modified list of biological methods (Part I) and biological methods and their validation criteria (Part II) of this document.

#### Recommendation

The Committee is invited to consider the modified list of biological methods (Part 1) and biological methods and their validation criteria (Part 11).

## PART I

### INTRODUCTION

The EWG started its work by considering the last point in paragraph 64 of REP16/MAS:

***“It was therefore suggested to revise the list and not to define criteria for the methods which might be removed from the list. A proposal could then be put to the relevant Codex Committee to review the methods and inform CCMAS whether they still wished to retain the biological methods”.***

So starting from the more recent list of the CODEX STAN 234-1999 (with the amendments adopted by the 39<sup>th</sup> Session of the Codex Alimentarius Commission in 2016) we modified the list of biological methods we proposed at the last CCMAS session.

We added two columns “Propose to remove or change Type” and “Possible method proposed”.

We took into account that the methods used to quantify vitamins by HPLC have been strongly improved in the last twenty years and they have nearly replaced all the old microbiological methods.

Some microbiological methods can be considered still useful for the quantification of vitamin B12, folates and pantothenic acid in foods. But in the coming years LC/MS should lead to remove or change type of microbiological methods.

#### Recommendation

The EWG proposes the following modified list of Biological methods:

### DETERMINATION OF FERMENTABILITY

Commodity	Provision	Method	Principle	Type	Propose to remove or Change	Possible method Proposed
Fruits juices and nectars	Determination of fermentability	IFUMA 18	Microbiological method	I	No	-----

**FOLIC ACID**

Commodity	Provision	Method	Principle	Type	Propose to remove or Change	Possible method Proposed
Special foods	Folic acid	AOAC 944.12	Microbioassay	II	No	-----
Infant formula	Folic acid	AOAC 992.05 (Measures free folic acid + free, unbound natural folates, aggregated and measured as folic acid) EN 14131 (Total folate (free + bound), aggregated and measured as folic acid)	Microbioassay	II	No	-----

**VITAMIN B3: NICOTINAMIDE**

Commodity	Provision	Method	Principle	Type	Propose to remove or Change	Possible method Proposed
Special foods	Nicotinamide for milk-based foods	AOAC 944.13	Microbioassay	II	Yes (III)	HPLC method like EN 15652 (Type II)

**VITAMIN B3: NIACIN**

Commodity	Provision	Method	Principle	Type	Propose to remove or Change	Possible method Proposed
Infant formula	Niacin	AOAC 985.34 (niacin (preformed) and nicotinamide)	Microbioassay And turbidimetry	III	No	HPLC method like EN 15652 (Type II)

**VITAMIN B5: PANTOTHENIC ACID**

Commodity	Provision	Method	Principle	Type	Propose to remove or Change	Possible method Proposed
Special foods	Pantothenic acid / enriched foods	AOAC 945.74	Microbioassay	II	No	-----
Special foods	Pantothenic acid / non-enriched foods	The Analyst 89 (1964): 1, 3-6, ibid.232 US DeptAgr., Agr.Handbook97 (1956)	Microbioassay	IV	No	-----
Follow-up formula	Pantothenic acid	AOAC 992.07 Measures total pantothenate : free pantothenic acid + bounded forms	Microbioassay	II	II or III	AOAC 2012.16/ISO 20639 UHPLC MS/MS (Type I or II)

**VITAMIN B6: PYRIDOXINE**

Commodity	Provision	Method	Principle	Type	Propose to remove or Change	Possible method Proposed
Infant formula	Vitamin B6	AOAC 985.32	Microbioassay	III	---	HPLC-Fluorescence like AOAC 2004.07 or EN 14164 (Type II)
Infant formula	Vitamin B6	CEN 14166 (Aggregates free and bound pyridoxal, pyridoxine and pyridoxine and measures as pyridoxine)	Microbioassay	III	----	HPLC – Fluorescence like AOAC 2004.07 or EN 14164 (Type II)
Special foods	Vitamin B6	AOAC 961.15	Microbioassay	II	type III	HPLC-Fluorescence like AOAC 2004.07 or EN 14164 (Type II) and EN 14663 (includes glycosylated forms) (Free and bound phosphorylated and glycosylated forms measured as the individual forms pyridoxal, pyridoxine and pyridoxamine), HPLC fluorometric method, (Type III)

**VITAMIN B12: COBALAMIN**

Commodity	Provision	Method	Principle	Type	Propose to remove or Change	Possible method Proposed
Special foods	Vitamin B12	AOAC 952.20	Microbioassay	II	Type III	HPLC-UV AOAC 2011.10 / ISO 20634 (Type II)
Infant Milk formula	Vitamin B12	AOAC 986.23	Bioassay-Turbidimetric	II	Type III	HPLC UV AOAC 2011.10 / ISO 20634 (Type II)

**VITAMIN D: ERGOCALCIFEROL (D2) & cholecalciferol (D3), OTHERS**

Comodity	Provision	Method	Principle	Type	Propose to remove or Change	Possible method Proposed
Margarine	Vitamin D	AOAC 936.14	Bioassay	II	Yes (Type III or II)	HPLC method like EN 12821 (Type II or III) AOAC 992.26 (D3) for HPLC-UV Type III AOAC 995.05 (D2&D3) for HPLC -UV (Type III)
Specialfoods	Vitamin D	AOAC 936.14	Rat bioassay	IV	----	HPLC methodlike EN 12821 (Type II)
Milk based Infant formula	Vitamin D2 and vitamin D3	-----	----	----	-----	Temporary ISO reference ISO/CD/20636 should become very soon the type II method (UPLC-MS/MS method)

**MARINE BIOTOXINS**

Commodity	Provision	Method	Principle	Type	Propose to remove or Change	Possible method Proposed
Live and raw bivalve molluscs	Paralytic shellfish toxicity	AOAC 959.08	Mouse bioassay	IV	III*	-----

Note 1: A Type III Method is one which meets the criteria required by the Codex Committee on Methods of Analysis and Sampling for methods that may be used for control, inspection or regulatory purposes. In the case of this method, it is said that it meets certain requirements, if it is left Type IV would mean that it did not fulfill any requirement. Both methods have an AOAC intercomparison validation that allowed them as biological methods to be used satisfactorily for control purposes.

Note 2:the Joint FAO/WHO Technical Paper 'Toxicity Equivalency Factors for Marine Biotoxins Associated with Bivalve Molluscs' :2016 (<http://www.fao.org/3/a-i5970e.pdf>) and within this Technical Paper Table 5.1 'TEFS Recommended for each Biotoxin Group by the Expert Group' plus specific guidance on how to apply TEFs to calculate total potency in a given sample (page 66 - 70); this information will allow the 'Toxin Analogues by Chemical Methods' - 'Applicable methods that meet the criteria' from CODEX STAN 234-1999 Table 1 page 16-17 to be cited as 'Possible method Proposed' for the 'Paralytic shellfish toxicity' Provision.

\*: Several countries propose that the method be changed to type II, because the MBA has parameters of accuracy and precision, LOD and intercomparisons. Therefore, according to the typification of CCMAS methods, it would not be type IV.

**PER**

Commodity	Provision	Method	Principle	Type	Propose to remove or Change	Possible method Proposed
Special foods	Protein efficiency ration (PER)	AOAC 960.48	Rat bioassay	I	No	-----

## PART II

**BIOLOGICAL METHODS AND THEIR VALIDATION CRITERIA**

**Bioassay:** Method in which potency of a substance is measured by the response of living organisms or living systems (such, cell-, receptor- or immunoassay based analysis tools).

**Bioassays classification based bioassays type:**

- **Qualitative bioassays** are those that do not generate a measurable graduated response, obtaining an absolute answer to the test unit. The bioassay gives a negative or positive response based on a specified concentration threshold.
- **Quantitative bioassays** produce a graduated response that generates a numeric value.

**FUNDAMENTALS OF BIOASSAY VALIDATION**

The goal of validation for a bioassay is to confirm the operating characteristics of the procedure for its intended use. Multiple dilutions (concentrations) of one or more Test samples and the Standard sample maybe included in a single bioassay.

These dilutions are herein termed a *replicate set*, which contains a single organism type, e.g., group of animals or vessel of cells, at each dilution for each sample [Test(s) and Standard].

In practice, a run frequently consists of the work performed by a single analyst in one lab, with one set of equipment, in a short period of time (typically a day). An *assay* is the body of data used to assess similarity and estimate potency relative to Standard for each Test sample in the assay.

**TERMS**

**Accuracy:** of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots homogeneous.

Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected study sample concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Accuracy is further subdivided into within-run, intr-batch precision (accuracy) or repeatability, with assesses precision(accuracy) during a single analytical run, and between run precision inter-batch precision or repeatability, with measures precision with time, and may involve different analysts, equipment, reagents, and laboratories.

Sample concentrations above the upper limit of the standard curve should be diluted. The accuracy and precision of these diluted samples should be demonstrated in the method validation.

**Biological Activity:** The specific ability or capacity of the product to achieve a defined biological effect.

**Biological matrix:** A discrete material of biological origin that can be sampled and processed in a reproducible manner.

**Potency:** The measure of the biological activity using a suitably quantitative biological assay (also called potency assay or bioassay), based on the attribute of the product which is linked to the relevant biological properties. Is the quantitative measure of the biological activity.

**Chart linearity**

Graph values obtained analytical response (y axis) as function of each of the added concentration values (x axis). Verify visually the existence of linearity of the data and verify the coefficient of determination R<sup>2</sup> which should be greater than 0.7.

**Coefficient of Variation (CV%):** Is a relative standard deviation. Coefficient of Variation is 100 times the ratio of the standard deviation to the mean, expressed as a percentage, e.g., a CV of 20% means that the standard deviation is 0.2 times the mean.

**Conversion Factor:** Conversion factor (CF value) expressing µg poison equivalent to 1 mouse unit (MU).

$$CF = \text{Concentration of STX (ug/ml)} / \text{MU (corrected)}$$

**Geometric standard deviation (%GSD):** 'Geometric standard deviation (GSD): The variability of the log-transformed values of a lognormal response expressed as a percentage in the untransformed scale. It is found as antilog(S), where S is the standard deviation determined in the log scale.

**Limit of detection (LOD):** The lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from background noise.

**Limit of quantification (LOQ):** Is the lowest concentration of analyte that can be determined with an acceptable level of uncertainty. It should be established using an appropriate measurement standard or sample, i.e. it is usually the lowest point on the calibration curve (excluding the blank). It should not be determined by extrapolation. *For a bioanalytical method, the reporting level shall be demonstrated to be different from procedure blank samples at least by a factor of three, with a response below the working range. It shall therefore be calculated from samples containing the target compounds around the required minimum level, and not from a S/N ratio or an assay blank.*

**Linearity** refers the ability of a test to generate directly proportional to the concentration of analyte in the sample results. Is obtained by preparation of triplicate and independently, sample blanks or spiked samples with a minimum of 5 different concentration levels of the provision of interest. To set the appropriate concentration range, considering the value of the specification, insofar as possible, as average level of that interval or the levels of the calibration curve. Analyze the samples prepared using the test method and determine the analytical response for each concentration level added.

**Residual plot.** Is made by calculate the slope (m) and intercept (b) and these data determine the value of (y') adjusted analytical response for each concentration value using the following equation:

$$y' = mx + b$$

Calculate the values residual (yy'), which is the difference between the value obtained (y) analytical response and calculated using the fitted curve (y') for each concentration level value.

Plotting the residual value (y axis) in function of the corresponding concentration (x axis). Verify data dispersion.

Report the linear range obtained accordance with the concentration units established by the method.

**Recovery:** Is the fraction or percentage of the analyte that is recovered when the test sample is conducted through the entire method. With the recovery data obtained in determining the operating range, determine the confidence interval% recovery. Report interval recovery obtained.

**Relative Accuracy (Relative bias= RB):** The *relative accuracy* of a relative potency bioassay is the relationship between measured relative potency and known relative potency. *Relative accuracy* in bioassay refers to a unit slope between log measured relative potency vs. log level when levels are known. The relative bias at individual levels is calculated as follows:

With the data obtained in the linearity test and from the analytical response obtained calculate the amount of vitamin for each of the added levels. Obtain the% recovery by the following formula:

$$\% \text{Recovery} = \frac{C_f}{C_a} * 100$$

Where:

$C_f$  = concentration of vitamin recovered white or spiked sample.

$C_a$  = concentration of vitamin added to the test sample.

Calculate the mean, standard deviation and relative standard deviation of each of the recovery values for each concentration level of vitamin.

Verify that all concentration levels meet the acceptance criteria for recovery and repeatability.

Plotting concentration data retrieved (y axis) as function of added concentration (x axis).

Calculate the correlation coefficient.

Report the working range obtained accordance with the concentration units established by the method.

**Reproducibility:** Precision obtained under observation conditions where independent test results are obtained with the same method on identical test in different test facilities with different operators using different equipment.

**Repeatability:** Precision obtained under observation conditions where independent test results are obtained with the same method on identical test items, in the same test facility by the same operator using the same equipment within short intervals of time.

**Specificity:** For products or intermediates associated with complex matrices, specificity (sometimes called selectivity) involves demonstrating lack of interference from matrix components or product-related components that can be expected to be present. This can be assessed via parallel dilution of the Standard sample with and

without a spike addition of the potentially interfering compound.

**Uncertainty:** Estimation that characterizes the range of values, within which is the conventionally true value of the measured magnitude.

**Work Range:** The scale of analysis of bioassay validation, where data are the relative potencies of samples in the validation study.

## RELEVANT PERFORMANCE CHARACTERISTICS OF BIOASSAY METHODS TO DETERMINATE OF VALIDATION

### Criteria of biological methods according to the analytes

Parameter	For Methods currently made official by the codex		
	Vitamins	Marine Biotoxins Paralytic shellfish toxin	PER
<b>Specificity</b>	Absence of the analyte in a free matrix of the same. Absence of the analyte in a free matrix of the same, Evidence that the substance quantified is the intended analyte. Each analyte is tested to ensure that there is no interference.	Absence of the analyte in a free matrix of the same and potential interfering substances.	Absence of the analyte in a free matrix of the same and potential interfering substances.
<b>Work Range</b>	Linearity At least 4 level correlation coefficient for > <b>0.97</b> and random distribution of points around the line. Range The correlation coefficient is greater <b>than 0.97</b> in the graph of concentration recovered against added concentration.	(One country proposes that the target range for the MBA is a death time of 5 to 7 min, this could be converted to STX equivalents).	-----
<b>Recovery</b>	70-130%	70-130%	-----
<b>RB (Bias)</b>	≤ 10%	≤ 10%	≤ 10%
<b>Limit</b>	LOD < LOQ < LMP	≤ 40 µg STX diHCl eq/100g	-----
<b>Repeatability RSD<sub>r</sub>%</b>	≤ 25%	-----	-----
<b>Intermediate Accuracy RSD<sub>R</sub>%</b>	≤ 30%	≤ 50%	-----
<b>Toxicologic parameter</b>	CPk TEQ TEF	CF: The CF values determined in routine controls should check with average CF within ± 20%. TEQ TEF	TEQ

**Bibliographic Reference:**

- 1 Technical paper. Toxicity Equivalency Factors for Marine Biotoxins Associated with Bivalve Molluscs. JOINT FAO/WHO. 2016.
- 2 Official Methods of Analysis of AOAC INTERNATIONAL, 20th Edition (2016).
- 3 Campos-Gimenez et al. Determination of vitamin B12 in infant formula and adult nutritionals by liquid Chromatography/UV Detection with Immunoaffinity Extraction: First Action 2011.08. Journal of AOAC International Vol 95 , N° 2, 2012.
- 4 Stevens & Dowell. Determination of Vitamins D2 and D3 in Infant Formula and Adult Nutritionals by Ultra-Pressure Liquid Chromatography with Tandem Mass Spectrometry Detection (UPLC-MS/MS): First Action 2011.12. Journal of AOAC International, Vol. 95, N° 3, 2012.
- 5 Staffas & Nyman. Determination of Cholecalciferol (Vitamin D3) in Selected Foods by Liquid Chromatography NMKL Collaborative Study. Journal of AOAC International, Vol. 86, N°. 2, 2003.
- 6 Determination of Vitamin D in foods: Current Knowledge and data gaps MPI technical paper N°. 2014/03. Ministry of Agriculture and Forestry under Project NUT/09/01 Science Programme Reporting, as part of overall contract for scientific services. Ministry for Primary Industries Manatū Ahu Matua.
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- 8 Guidance for Industry Bioanalytical Method Validation U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine (CVM) May 2001.
- 9 The Fitness for Purpose of Analytical Methods A Laboratory Guide to Method Validation and Related Topics. Second Edition 2014. EURACHEN.
- 10 Commission Regulation (EU) No 589/2014 of 2 June 2014 laying down methods of sampling and analysis for the control of levels of dioxins, dioxin-like PCBs and non-dioxin-like PCBs in certain foodstuffs and repealing Regulation (EU) No 252/2012
- 11 Establishing Acceptance Criteria for Analytical Methods Knowing how method performance impacts out-of-specification rates may improve quality risk management and product knowledge. Analytical Best Practices.
- 12 ICH Harmonised tripartite guideline specifications: test procedures and acceptance criteria for biotechnological/biological products Q6B. International Conference On Harmonisation Of Technical Requirements For Registration Of Pharmaceuticals For Human Use. 1999.
- 13 Appendix k: guidelines for dietary supplements and botanicals. Aoac.
- 14 Step-by-step analytical methods validation and protocol in the quality system compliance industry. By Ghulam A. Shabir.
- 15 TG455: draft performance-based test guideline for stably transfected transactivation in vitro assays to detect estrogen receptor agonist and antagonists. OECD/OCDE guideline for testing of chemicals. 2016.
- 16 NB: Version adopted by the World Assembly of Delegates of the OIE in May 2013. OIE *Terrestrial Manual* 2013 1 Chapter 1.1.5. Principles And Methods Of Validation Of Diagnostic Assays For Infectious Diseases.
- 17 OECD Guideline For The Testing Of Chemicals. The Hershberger Bioassay in Rats.
- 18 Peter A. Behnisch et al. Harmonised Quality Criteria For Chemical And Bioassays. Analyses Of PCDDs/PCDFs In Feed And Food. Part 2: General Considerations, Bioassay Methods.
- 19 AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals.

## APPENDIX I

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