ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	**DSP PPIA Kit for Determinination of Okadaic Acid Toxins Group (OA, DTX1, DTX2) in Molluscan Shellfish**
Name of the Method Developer	**Zeu-Inmunotec S.L.**
Developer Contact Information	**David C. Deardorff / Elena Dominguez Abraxis LLC / Zeu-Inmunotec S.L. 54 Steamwhistle Drive / Poligono Plaza, C/ Bari 25, Warminster, PA 18974 / 50197 Zaragoza USA / Spain**

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	The method is a rapid, simple and reliable method for ocadaic acid and related toxins analysis in shellfish for the industry sector. The method is easy to set up and therefore accessible for small test facilities close to the site of operation.
2. What is the intended purpose of the method?	Y	The method was developed and validated at the SLV level for the determination of okadaic acid and related toxins in clams, oysters and mussels.
3. Is there an acknowledged need for this method in the NSSP?	Y	The supply of rapid, simple, reliable and easily accessible method alternatives to demanding liquid chromatography methods will enable the shellfish industry to employ local test facilities close to the site of operation. This will again enable preventative countermeasures and immediate response to elevated OA levels locally, significantly reducing the risk of exposing the consumers to contaminated shellfish.
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	Protein Phosphatase Inhibition Assay (PPIA)
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	DSP PPIA Kit for Determination of Okadaic Acid Toxins Group (OA, DTX1, DTX2) in Molluscan Shellfish
Method Scope	Y	For the determination of ocadaic acid and related toxins in shellfish.
References	Y	1) Smienk et al. (2013) Quantitative Determination of the Okadaic Acid Toxins Group by a Colorimetric Phosphtase Inhibition Assay: Interlaboratory Study. *J. AOAC Intl.* **96**; 77-85 2) Smienk et al. (2012) Single Laboratory Validation of A Ready-To-Use Phosphatase Inhibition Assay for Detection of Okadaic Acid Toxins. *Toxins* **6**, 339-352

		3) Zeu-Inmunotec S.L. (2011) Collaborative study to validate a colorimetric phosphatase inhibition assay for determination of OA-toxins group in Mollusks: TOXILINE-DSP.Co, *Validation Report G-COM-OA 10. Rev. 1.* Zeu-Inmunotec S.L., Zaragoza, Spain (www.zeulab.com) 4) Zeu-Inmunotec S.L. (2010) Single Lab Validation Report *SLV-OkaTest, G-COM-OA 09. Rev. 3.* Zeu-Inmunotec S.L., Zaragoza, Spain (www.zeulab.com) 5)a Abraxis LLC (2012) *PN 520025 - Okadaic Acid (PP2A), Micotiter Plate User's Guide.* Abraxis LLC, Warminster, PA (www.abraxiskits.com) b Zeu-Immunotec S.L. (2010) *PN ZE/OA96C - OkaTest User's Guide.* Zeu-Inmunotec S.L., Zaragoza, Spain (www.zeulab.com)
Principle	Y	The kit is based on the phosphatase activity inhibition by oa-toxins group, responsible for diarrheic shellfish poisoning (DSP). Phosphatase enzyme PP2A is able to hydrolyse a specific substrate, yielding a product that can be detected at 405 nm. Samples containg toxins from the okadaic group will inhibit the enzyme activity proportionally to the amount of toxin contained in the sample. The concentration of toxin in the sample can be calculated using a standard curve.
Any Proprietary Aspects	Y	Unique PP2A enzyme
Equipment Required	Y	Specified in the user's guides (ref 5)
Reagents Required	Y	Specified in the user's guides (ref 5)
Sample Collection, Preservation and Storage Requirements	Y	Sample preparation procedure is specified in the user's guides. Kit storage requirements are specified in the user's guides. The kit is stable for 6 months after production when stored at 4C
Safety Requirements	Y	Safety precautions are specified in the user's guides.
Clear and Easy to Follow Step-by-Step Procedure	Y	Specified in the user's guides as a detailed procedure and a quick reference flow chart
Quality Control Steps Specific for this Method	Y	Quality assurance measures are specified in the user's guides.
C. Validation Criteria		
1. Accuracy / Trueness	Y	The accuracy/trueness was determined and reported in the first four cited references.
2. Measurement Uncertainty	Y	The uncertainty is described in paragraph 3.10 of the SLV report (ref 4)
3. Precision Characteristics (repeatability and reproducibility)	Y	The repeatability precision was determined and reported in the first four cited references.
4. Recovery	Y	The recovery/accuracy was determined and reported in the first four cited references.
5. Specificity	N	
6. Working and Linear Ranges	Y	Both the working and linear range of the assay is 0.5 to 2.8 nM OA; 63 to 352 ug OA equivalents/kg (OA, DTX1, DTX2 and ester forms)
7. Limit of Detection	Y	The shellfish LOD is 44 ug/Kg
8. Limit of Quantitation / Sensitivity	Y	The shellfish LOQ is 0.5nM or 63 ug/Kg
9. Ruggedness	Y	A ruggedness study is described in paragraph 3.3 of the SLV study(ref 2) and paragraph 3.4 of the SLV report (ref 4)
10. Matrix Effects	Y	The selectivity has been documented with structural analogues and the matrix affects have been examined in

		several shellfish as reported in the first four cited references.

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	Test results comparability to LC-MS/MS and MBA methods has been reported in paragraph 3.7 of the SLV study(ref 2) and Table 23 of the SLV report(ref 4).
D. Other Information		
1. Cost of the Method	Y	$600 for 43 duplicate results ($13.95/sample)
2. Special Technical Skills Required to Perform the Method	Y	Some technical skills are required. Familiarity with laboratory procedures is desired. On-site training and visual aids are available.
3. Special Equipment Required and Associated Cost	Y	A microwell strip/plate reader w/405nm filter and manual pipettes are required. The estimated costs range from $1,800 to $8,000 for manual to automatic features.
4. Abbreviations and Acronyms Defined	Y	PPIA; Protein Phosphatase Inhibition Assay OA: Okadaic Acid DSP: Diarrheic Shellfish Poisons
5. Details of Turn Around Times (time involved to complete the method)	Y	The sample preparation will depend on the number of samples. 10 samples can be prepared in 60 minutes. 43 prepared samples can be analyzed in under 90 minutes.
6. Provide Brief Overview of the Quality Systems Used in the Lab	Y	The quality policy contains a) manufacturing quality system, b) documentation control system, c) written master batch record including master formula, labeling and manufacturing SOPs, d) individual batch records which are maintained and kept through product expiration, e) product performance testing requirements which are conducted on each batch, f) unique lot numbers for each batch which are traceable from raw materials through finished products, g) a stability program, h) a quality audit function, and i) a mechanism for disposition of non-conforming materials.
Submitters Signature	Date: 28 June 2013	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	
Accepted	Date:	
Recommendations for Further Work	Date:	
Comments:		

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection - the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity - the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range - the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix - The component or substrate of a test sample.
12. Method Validation - The process of verifying that a method is fit for purpose.[1]
13. Precision - the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability - the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility - the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. Quality System - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. Recovery - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. Ruggedness - the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. Specificity - the ability of a method to measure only what it is intended to measure.[1]
18. Working Range - the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.

3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

FOOD CHEMICAL CONTAMINANTS

Quantitative Determination of the Okadaic Acid Toxins Group by a Colorimetric Phosphatase Inhibition Assay: Interlaboratory Study

HENRY SMIENK and ELENA DOMÍNGUEZ[1]
ZEU-INMUNOTEC, C/Bari 25 Dpdo. 50197, Zaragoza, Spain
MARÍA L. RODRÍGUEZ-VELASCO
EURLMB-AESAN, Estación Marítima S/N, Muelle de Trasatlánticos, 36200 Vigo, Spain
DAVID CLARKE
Marine Institute, Rinville, Oranmore, Co. Galway, Ireland
KATRIN KAPP
BFR—Federal Institute for Risk Assessment, Thielallee 88-92, 14195, Berlin, Germany
PANAGIOTA KATIKOU
NRL Marine Biotoxins, Institute of Food Hygiene, 3A Limnou St, 54627 Thessaloniki, Greece
ANA G. CABADO, ALBERTO OTERO, and JUAN M. VIEITES
ANFACO CECOPESCA, Ctra. Colexio Universitario, 16, Lagoas, 36310, Vigo, Spain
PEDRO RAZQUIN and LUIS MATA
ZEU-INMUNOTEC, C/Bari 25 Dpdo., 50197, Zaragoza, Spain

An interlaboratory collaborative study to validate a colorimetric phosphatase inhibition assay for quantitative determination of the okadaic acid (OA) toxins group in molluscs, OkaTest, was conducted. Eight test materials, including mussels, scallops, clams, and cockles, were analyzed as blind duplicates. Blank samples and materials containing different OA toxin levels ranging from 98 to 275 µg/kg OA equivalents were included. The study was carried out by a total of 16 laboratories from 11 different countries. Values obtained for repeatability relative standard deviations (RSD_r) ranged from 5.4 to 11.2% (mean 7.5%). Reproducibility RSD (RSD_R) values were between 7.6 and 13.2% (mean 9.9%). The Horwitz ratio (HorRat) values ranged between 0.4 and 0.6. A recovery assay was also carried out using a sample spiked with OA. A mean recovery of 98.0% and an RSD of 14.5% were obtained. The results obtained in this validation study indicate that the colorimetric phosphatase inhibition assay, OkaTest, is suitable for quantitative determination of the OA toxins group. OkaTest could be used as a test that is complementary to the reference method for monitoring the OA toxins group.

Okadaic acid (OA) and its analogs dinophysistoxin-1 and -2 (DTX1, DTX2), together with their ester forms, are known as the OA toxins group. These lipophilic and heat stable toxins are produced by dinoflagellates and can be found in various species of shellfish, mainly in filter-feeding bivalve molluscs.

OA toxins causes diarrheic shellfish poisoning, which is characterized by symptoms, such as diarrhea, nausea, vomiting, and abdominal pain. These symptoms may occur in humans shortly after consumption of contaminated bivalve molluscs, such as mussels, clams, scallops, or oysters. Inhibition of serine/threonine phosphoprotein phosphatases (PPs) is assumed to be responsible for these toxic effects. These compounds are also involved in tumor promotion (1). Therefore, these toxins are regulated by European Union law.

Regulation (EC) No. 853/2004 (2) states that live bivalve molluscs placed on the market for human consumption must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed 160 µg of OA equivalents/kg for OA, dinophysistoxins, and pectenotoxins together.

Commission Regulation (EC) No. 15/2011 (3) indicates that in the case of lipophilic toxins including OA toxins, LC/MS/MS is the reference method for routine testing of official controls or any checks done by food operators. This regulation has recently amended the Commission Regulation (EC) No. 2074/2005 (4), in which biological methods (mouse and rat bioassay) were considered the reference. From now on, they will only be used for a transitional period of time (until the end of 2014) or in special circumstances.

Both regulations (No. 2074/2005 and No. 15/2011) contemplate other methods for routine testing of lipophilic toxins, providing they are intralaboratory-validated and successfully tested under a recognized proficiency test scheme. Those methods should detect, either alone or in combination with others, all of the lipophilic toxin analogs (OA, pectenotoxins, yesotoxins, and azaspiracids group toxins). The protein phosphatase inhibition assay (PPIA) is specifically mentioned in these regulations as an alternative or complementary method, considering that the PPs are known to be OA-toxins natural targets (5, 6). In-house PPIAs using different phosphatase sources and colorimetric or fluorometric substrates have been previously developed (7–12). Later improvements to detect all OA derivatives by hydrolysis of samples were also suggested

Received October 28, 2011. Accepted by AP May 23, 2012.
[1] Corresponding author's e-mail: edominguez@zeulab.com
DOI: 10.5740/jaoacint.11-465

Table 1. Details of matrixes and species origin of test materials used in this study

Code	Matrix/Species	Origin
A	Mussel (*M. galloprovincialis*)	Galicia (NW Spain)
D	Clam (*V. pullastra*)	Food & Agricultural Organization, 37 Mediterranean Sea
E	Mussel (*M. galloprovincialis*)	Galicia (NW Spain)
F	Scallop (*P. maximus*)	FAO 27 NE Atlantic
G	Clam (*V. decussatus*)	Galicia (NW Spain)
K	Clam (*V. romboides*)	Galicia (NW Spain)
L	Cockle (*C. edulis*)	Portugal and Galicia (NW Spain)
N	Mussel (*M. edulis*)	Ireland
BM	Scallop (*P. maximus*)	Scotland

(13), and a collaborative study was also performed with a fluorometric PPIA (14). However, none of those assays was commercially available for routine analysis, nor were they demonstrated to comply with the legislation requirements.

ZEU-INMUNOTEC (Zaragoza, Spain) has developed a commercial kit (OkaTest, formerly Toxiline-DSP) based on a colorimetric PP2A inhibition assay for quantification of the OA toxins group in molluscs (15).

The PPIA described in this study uses a human PP2A purified by ZEU-INMUNOTEC that has showed higher sensitivity than other commercial and genetic engineering produced enzymes (16). PP2A was stabilized by freeze-drying to obtain a standardized assay with shelf life of up to 12 months at 4°C (15). Colorimetric substrate was chosen over a fluorometric one as the latter is less stable and, therefore, less appropriate for ready-to-use kits. Besides, fluorometric assays require specific equipment not often available in routine testing laboratories; therefore, they are difficult to use for monitoring purposes.

The robustness and performance of OkaTest were evaluated by the manufacturer in a single-laboratory validation according to AOAC and Eurachem guidelines (15). All of the results obtained showed that the OkaTest kit is robust and accurate, and, therefore, suitable for an interlaboratory study.

Interlaboratory Study

A colorimetric PPIA, OkaTest, was interlaboratory-validated for quantification of the OA toxins group. The main purpose of this study was to determine repeatability and between-laboratory reproducibility. A recovery assay was also carried out, and accuracy of the method confirmed.

A validation management team (David Clarke, Elena Domínguez, Katrin Kapp, Panagiota Katikou, and María Luisa Rodríguez) was appointed to supervise, advise on the accomplishment of the study, and ensure its independence. A total of 16 laboratories from 11 different countries in Europe and South America participated in the study.

The study plan including details of the test method, experimental design, preparation of test materials, instructions for participants, key personnel, schedule, and data analysis was prepared and agreed to by the validation management team. Participants were fully informed of the study design prior to distribution of testing materials.

Eight different test materials, as blind duplicates, were analyzed by each laboratory on 2 different days. Five materials contained different OA toxin levels, all naturally contaminated except for one that was partially spiked. Three of the test materials were blank samples. An additional blank material (BM) was used in the recovery study. The test materials comprised four different genera of molluscs (*Mytilus spp, Pecten spp., Venerupis spp.,* and *Cerastoderma spp*.) and seven different species. Details of the materials used are shown in Table 1. The materials were prepared by the Spanish Association of Seafood Products Manufacturers (ANFACO-CECOPESCA; Vigo, Spain) as explained below.

All participants sent back an electronic copy of a tailor-made Excel reporting sheet for each day of analysis with raw data and final results for each test material. The reporting sheets were checked upon receipt for obvious errors in sample codes and calculations.

Participants also completed a questionnaire with details of the equipment used and preparation of reagents and samples, as well as feedback on the assay.

Preparation of Test Materials

Materials A and E (mussel) and D and K (clam) were purchased from the retail market fresh and alive. They were thoroughly cleaned outside and inside with fresh water to remove sand and any other foreign materials. Tissues were removed from the shell, transferred to strainers, and drained for 5 min before homogenization (blender and Ultraturrax®; IKA, Staufen, Germany). The homogenate (at least 450 g) was then distributed into plastic containers (5.0 ± 0.1 g), frozen, and stored at –20 ± 2°C until analysis or the day of shipment.

Materials F (scallop) and G (clam) were purchased frozen

Table 2. Total concentration of OA toxins group (µg/kg) determined by OkaTest, and toxins profile by LC/MS/MS

Test material[a]	Matrix/species	Total OA equivalents, µg/kg[b]	OA toxins content[c]
BM	Scallop (*P. maximus*)	<LOD	—
A	Mussel (*M. galloprovincialis*)	<LOD	—
F	Scallop (*P. maximus*)	<LOD	—
G	Clam (*V. decussatus*)	<LOD	—
E	Mussel (*M. galloprovincialis*)	79 ± 5	OA
L	Cockle[d] (*C. edulis*)	168 ± 11	OA, DTX1, and DTX2
D	Clam (*V. pullastra*)	240 ± 9	OA
K	Clam (*V. romboides*)	250 ± 6	OA
N	Mussel[e] (*M. edulis*)	276 ± 6	OA and DTX2

[a] Samples presented in increasing order of concentration.

[b] Determined by OkaTest; LOD = 44 OA equivalents µg/kg.

[c] Determined by LC/MS/MS.

[d] Artificially contaminated with DTX1 and mixed with blank material.

[e] Mixed with blank material.

Table 3. Results from homogeneity study for test materials for the determination of OA (µg OA total equivalents/kg)

Test material	Variance of sums, Vs	Analytical variance, s_{an}^2	Allowable sampling variance, σ_{all} ^2	Sampling variance, S_{sam}^2	Critical value, c	Test for homogeneity result
D	166	90.7	36.8	116	310	S_{sam}^2 < c
E	84.7	8.09	19.8	11.1	29.1	S_{sam}^2 < c
K	139	19.6	32.5	126	257	S_{sam}^2 < c
L	356	46.9	85.7	55.6	152	S_{sam}^2 < c
N	124	24.2	28.4	154	314	S_{sam}^2 < c

from the retail market. They were thawed at room temperature, cleaned, and prepared as described above.

Material L (cockle) was provided cleaned, blended, and frozen by the European Reference Laboratory for Marine Biotoxins (EURLMB, Vigo, Spain). The sample contained OA, DTX2, and traces of DTX1. In order to achieve a suitable toxin profile, the sample was mixed with fresh cockle from the same species (*C. edulis*) without toxin prior to being spiked with DTX1 (Wako Chemicals, Neuss, Germany). The sample was thawed at room temperature, mixed with the cockle blank material (purchased in Porto, Portugal), and spiked. Then, it was distributed into plastic containers (5.0 ± 0.1 g), frozen, and stored at –20 ± 2°C until the day of shipment.

Material N (mussel) was provided cleaned, blended, and frozen by the National Reference Laboratory of Ireland, Galway, Ireland. The sample contained a high level of OA toxins, so it was mixed with mussel (*M. edulis*) without toxin (purchased in a retail market in Ireland) to achieve a suitable toxin concentration. The sample was thawed at room temperature, mixed, and distributed into plastic containers (5.0 ± 0.1 g). The material was then frozen and stored at –20 ± 2°C until the day of shipment.

The BM (scallop) was provided blended and homogenized by Integrin Advanced Bioscience (Oban, Scotland) and stored frozen at approximately –20 ± 2°C until the day of shipment.

Homogeneity and stability of test materials were studied according to the International Harmonized Protocol for the Proficiency Testing of Analytical Chemistry Laboratories (17). Ten containers of 5 g were randomly selected for each material. The content of each container was homogenized and extracted, and two test portions (from the sample extract) were analyzed to estimate the analytical variance. A total of 20 portions/material were tested under repeatability conditions and in a random order using the OkaTest kit.

To ensure the stability of the materials during shipment to participants and the study duration, aliquots of each material were taken randomly and split into two subsets, each of them containing five samples. One subset was used as control and stored at –18 ± 1°C. The second was stored under experimental conditions of 9.0 ± 1°C for 5 days. Samples of both subsets were randomized before testing and analysis simultaneously using the OkaTest kit under repeatability conditions. The test materials were also analyzed by LC/MS/MS (18, 19) to determine the OA toxin profile.

The test materials were blind coded by EURLMB and distributed by ANFACO-CECOPESCA to the participants. The codes were securely kept by EURLMB until statistical analysis was carried out.

The materials were shipped in isothermal boxes with dry ice and were received within the following 2 days by most participants. Materials sent to South American countries were delivered more than a week after the dispatch date, as they have long customs check up procedures. Samples were, however, reported to have been kept frozen while stored at customs. Two laboratories informed that the box containing the samples did not arrive in good conditions, and six reported that samples were cold, but defrosted.

PPIA

Principle

OkaTest is an enzymatic test based on a colorimetric PPIA for quantitative determination of OA and other toxins of the OA group, including DTX1, DTX2, and their ester forms.

Table 4. Results obtained for the stability assays conducted for materials D, E, K, L, and N

	Storage conditions						
	–18 ± 1°C	9.0 ± 1°C					
	Mean						
Test material	Total OA equivalents, µg/kg		Absolute difference D	Variance *F*-test	*t*-test	Test criterion C	D < C
D	265 ± 10	262 ± 15	3.02	0.54	0.71	34.5	Pass
E	84.0 ± 4	85.1 ± 3	–1.19	0.45	0.62	10.9	Pass
K	255 ± 8	257 ± 7	–1.57	0.87	0.75	33.2	Pass
L	171 ± 7	169 ± 8	1.63	0.79	0.73	22.2	Pass
N	343 ± 24	355 ± 32	–13.0	0.58	0.49	44.6	Pass

Table 5. Calibration curve parameters obtained by each laboratory every day of the study

	R^2		Slope		Absorbance 405 nm, lowest standard 0.5 nM		Absorbance 405 nm, highest standard 2.8 nm	
Lab	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
A	0.99	0.98	–0.12	–0.45	0.734	1.287	0.524	0.505
B	0.99	0.99	–0.50	–0.65	1.157	1.425	0.334	0.339
C	0.98	0.98	–0.64	–0.44	1.530	1.177	0.496	0.468
D	0.98	0.98	–0.67	–0.58	1.537	1.402	0.430	0.459
E	0.97	0.98	–0.51	–0.48	1.222	1.221	0.409	0.436
F	1.00	0.99	–0.72	–0.74	1.684	1.726	0.482	0.491
G	0.98	1.00	–0.79	–0.58	1.781	1.411	0.462	0.423
H	0.99	0.99	–0.78	–0.73	1.644	1.609	0.366	0.414
I	0.99	0.99	–0.76	–0.68	1.661	1.486	0.409	0.357
J	0.97	0.98	–0.41	–0.45	1.164	1.204	0.498	0.458
K	0.99	0.98	–0.77	–0.74	1.712	1.690	0,438	0,485
L[a]	0.93	0.96	–0.63	–1.13	1.488	2.588	0.425	0.709
M	0.99	0.99	–0.78	–0.65	1.697	1,464	0.419	0.390
N	0.99	0.98	–0.54	–0.65	1.273	1,497	0.384	0.444
O	0.97	0.98	–0.49	–0.32	1.188	0,992	0,396	0.470
P	0.97	0.99	–0.27	–0.58	1.015	1.474	0.549	0.520

[a] Standard curve obtained by Laboratory L on Day 1 was rejected as R^2 criterion was not met. Assay could not be repeated due to time issues.

This method is applicable to shellfish species, such as mussels, clams, cockles, and scallops.

The toxicity of the OA toxins group is directly related to its inhibitory activity against a family of structurally related PPs, in particular PP1 and PP2A. OkaTest uses this strong inhibitory activity to determine the OA content in shellfish using the PP2A with a chromogenic substrate for this enzyme. After the substrate's hydrolysis by the enzyme, the product can be measured at 405 nm by a microplate reader. As the ability of the PPs to hydrolyze the substrate depends on the amount of OA and analogs in the samples, the toxin concentration can be calculated by using a standard curve.

Apparatus

(**a**) *Micropipets*.—Adjustable 100, 200, and 1000 µL (Thermo Labsystems, Helsinki, Finland).

(**b**) *Ultra homogenizer*.

(**c**) *Block heater or incubator*.—For 30 ± 2°C (ZEU-INMUNOTEC, Zaragoza, Spain).

(**d**) *Microwell absorbance reader*.—405 ± 10 nm wavelength filter (Thermo Labsystems).

(**e**) *Water bath*.—Set at 76 ± 2°C (Raypa, Barcelona, Spain).

(**f**) *Centrifuge tubes*.—Graduated 50 mL.

(**g**) *Laboratory glassware*.

Reagents

(**a**) *Extraction solvent*.—Methanol, reagent grade, 100% (v/v; Sharlab, Barcelona, Spain).

(**b**) *HCl*.—Reagent grade, 37% (v/v; Sharlab).

(**c**) *NaOH*.—Reagent grade (Sharlab).

(**d**) *Deionized water*.—Type II, ISO 3696 (Ellix 5; Millipore, Germany).

(**e**) *OkaTest kit*.—From ZEU-INMUNOTEC containing:

(*1*) 96-well microtiter plate and plate adhesive film.

(*2*) Lyophilized PP2A purified from human blood cells.

(*3*) Ready-to-use OA Standards of 0.5, 0.8, 1.2, 1.8, and 2.8 nM, prepared from the OA reference solution (NRC CRM-OA-c, Institute for Marine Biosciences, Halifax, Canada).

(*4*) Chromogenic substrate.

(*5*) Phosphatase dilution buffer.

(*6*) Stock buffer solution.

(*7*) OA Spiking solution (2 µM) prepared from the OA reference solution (NRC CRM-OA-c, Institute for Marine Biosciences).

Spiking Procedure

Due to the limited experience on the homogeneity and stability of spiked samples with OA toxins, each participant prepared a spiked sample on the day of the assay. A BM and an OA solution of known concentration (2 µM, to prepare a final concentration of 161 µg/kg) were provided to each participant.

A blank sample was spiked with OA solution for the recovery study as follows:

(**a**) Mix 500 µL OA spiking solution (2 µM) with 5.0 ± 0.1 g homogenous blank sample.

(**b**) Add 25 mL extraction solvent [methanol, 100% (v/v)] to the mixture and shake for 2 min by vortexing. Proceed with the extraction procedure described below under point (**b**).

Sample Extraction

(**a**) Thaw each aliquot with 5.0 ± 0.1 g homogenized mollusc at room temperature (22 ± 2°C). Add 25 mL extraction solvent [methanol, 100% (v/v)]; then mix for 2 min using an ultra homogenizer.

(**b**) Centrifuge at 2000 g for 10 min at 4°C. The supernatant is called "methanolic extract."

(**c**) Pipet 640 µL methanolic extract into a 50 mL graduated centrifuge tube and add 100 µL 2.5 M NaOH.

(**d**) Seal the test tube and heat at 76 ± 2°C for 40 min in a water bath.

(**e**) Do not cool the sample; add 80 µL 2.5 M HCl immediately.

(**f**) Add 19.18 mL buffer solution with a glass pipet up to a total volume of 20 mL.

Assay Procedure

(**a**) Rehydrate the lyophilized phosphatase (PP2A) by adding 2.0 mL phosphatase dilution buffer to the vial and mix gently for 60 ± 5 min at room temperature (22 ± 2°C) on a roller mixer or a shaker (maximum 60 rpm) (both from JP Selecta, Barcelona, Spain).

(**b**) Add 50 µL each sample extract or standard to wells. Samples and standards have to be analyzed in duplicate.

(**c**) Add 70 µL phosphatase solution to each well. Cover the plate with the adhesive film provided in the kit, and mix by gentle tapping on the side.

(**d**) Incubate at 30 ± 2°C for 20 ± 0.5 min.

(**e**) Remove the adhesive film and add 90 µL chromogenic substrate to each well and mix by tapping gently on the side. Incubate at 30 ± 2°C for 30 ± 0.5 min.

(**f**) Read the absorbance of samples and standards at 405 ± 10 nm.

Calculations

The results were calculated from a standard curve by plotting the absorbance values on a linear y axis and the concentration of OA on a logarithmic x axis, and using a logarithmic fitting. As an acceptability criterion for the assay, the Pearson correlation coefficient R^2 had to be equal to or greater than 0.96. The OA concentration contained in the sample was then calculated using the following equation:

$$x = EXP\ (y - b)/a$$

where x is the OA concentration in the sample (Cs), y the absorbance of the sample, a is the slope, and b is the y-intercept.

The OA toxin concentration in shellfish tissue was calculated as follows:

$$Ct,\ \mu g/kg = [Cs\ (nM) \times FD \times MW\ (g/mol) \times Ve\ (L)]/\ Mt\ (g)$$

where Ct is the toxin concentration in tissue expressed as equivalents of OA, FD is the methanolic extract dilution factor, MW of OA = 805, Ve is the methanolic extract volume (0.025 L), and Mt is the tissue weight (5 g).

Samples with an OA concentration falling outside the working range (<0.5 nM or >2.8 nM) will be reported as <63 µg/kg (or <0.5 nM) or >352 µg/kg (or >2.8 nM), respectively.

Results were recorded by each participant in a tailor-made Excel spreadsheet with which the results were automatically calculated when the absorbance values were entered. All participants sent back an electronic copy of the reporting sheet for each day of analysis.

Table 6. Individual results (µg OA total equivalents/kg) reported from laboratories A to P for Materials A, D, E, F, G, K, L, and N on Days 1 and 2. Invalid or incorrect results are those in bold type.

	µg OA total equivalents/kg															
	Material															
	A		D		E		F		G		K		L		N	
	Day															
Lab	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
A	<63	<63	186	239	97	102	<63	<63	<63	<63	248	281	167	174	210	247
B	<63	<63	251	266	100	101	<63	<63	<63	<63	302	299	177	190	273	277
C	<63	<63	244	233	96	87	<63	<63	<63	<63	279	246	174	160	256	251
D	<63	<63	264	253	125	100	<63	<63	<63	<63	282	277	189	223	269	295
E	<63	<63	210	233	101	120	<63	<63	<63	<63	239	244	156	181	226	219
F	<63	<63	252	250	113	116	<63	<63	<63	<63	287	286	166	165	271	275
G	<63	<63	246	252	89	100	<63	<63	<63	<63	356[a]	269[a]	192	192	274	236
H	<63	<63	253	250	90	99	<63	<63	<63	<63	291	301	175	179	271	270
I	<63	<63	252	254	95	87	<63	<63	<63	<63	284	283	169	161	265	253
J	70[a]	98[a]	238	239	163[a]	102[a]	<63	<63	78[a]	67[a]	248	268	239	184	246	235
K	<63	<63	253	264	81	81	<63	<63	<63	<63	295	300	152	160	247	266
L	**—**	**<63**	**—**	**242**	**—**	**145**	**—**	**<63**	**—**	**—**	**—**	**266**	**—**	**202**	**—**	**182**
M	<63	<63	257	255	101	104	<63	<63	<63	<63	292	274	177	176	271	272
N	<63	<63	261	251	98	101	<63	<63	<63	<63	285	285	161	181	257	250
O	<63	<63	221	223	91	94	<63	<63	<63	<63	270	249	179	184	259	244
P	<63	<63	192	241	69[a]	153[a]	<63	<63	<63	<63	226	278	97	173	206	259

[a] Outlier.

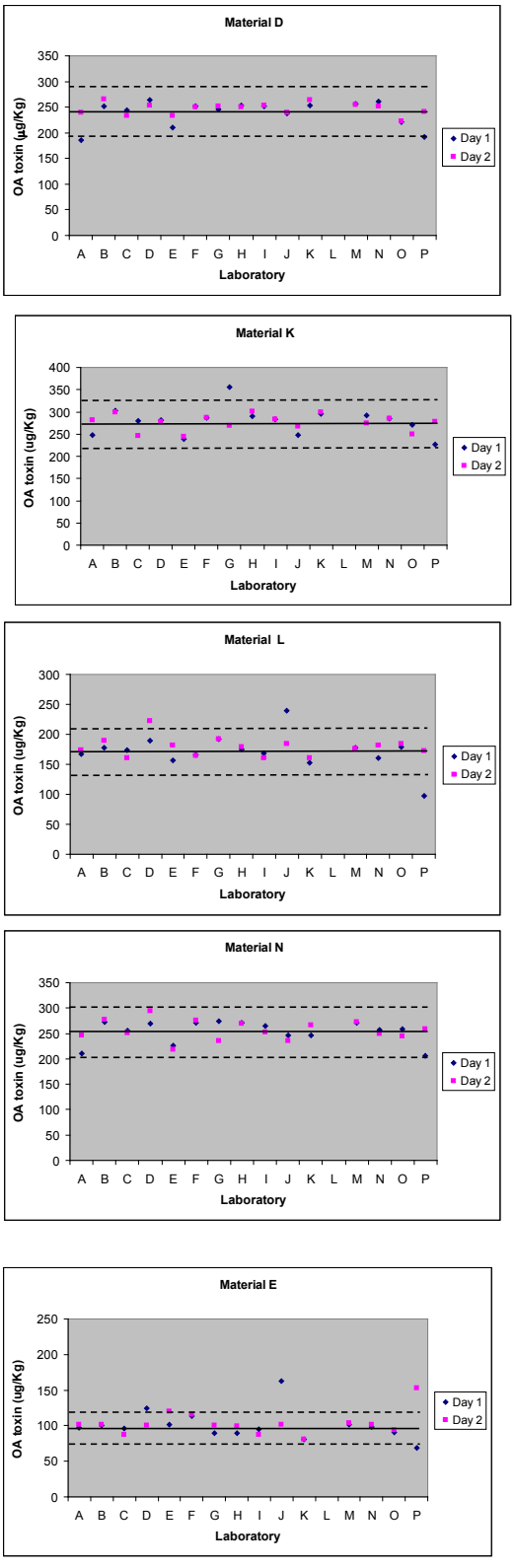


Figure 1. Individual results for each test material obtained per lab and per day of analysis (including outliers). The solid line shows the assigned mean value calculated in this study for each material. The dashed lines indicate the theoretical reproducibility SD determined for each material in this study ($PRSD_R$).

Statistics

Analysis of Valid Data and Outliers

Statistical data analysis was carried out following the approach described in the AOAC/IUPAC guidelines (17, 20). Submitted results were initially reviewed to remove invalid data. Results from assays with calibration curves with a $R^2 < 0.96$ and results outside the working range or showing deviations from the Standard Operating Procedure were considered invalid.

The valid data were first analyzed for possible outliers applying the Cochran and Grubbs tests. Then, precision parameters, HorRat values, and recovery were calculated.

The Cochran test was applied to remove laboratories showing significantly greater variability among replicate (within-laboratory) analyses than the other laboratories for a given material. A 1-tail test at a probability value of 2.5% was applied (17, 20).

The Grubbs test was used to remove results from laboratories with extreme averages (17, 20). This test was applied to the remaining values from the Cochran test. A single value test (two-tail, $P = 2.5\%$) was first applied, followed by a pair value test (two values at the highest end, two at the lowest end, and one at each end, at an overall $P = 2.5\%$).

Precision

To estimate the precision of the method, the within-laboratory repeatability and between-laboratory reproducibility were determined by calculating s_r (repeatability SD), s_R (reproducibility SD), RSDs (RSD_r and RSD_R), repeatability and reproducibility limits (r and R), and HorRat values. These parameters were calculated following the AOAC guidelines (20).

Recovery

For recovery calculations, the marginal recovery was calculated as follows:

$$\text{Recovery, \%} = 100\,(C_f - C_u)/C_A),$$

where C_f is the amount found for the spiked concentration, C_u is the amount present originally for the unspiked concentration, and C_A is the amount added.

Results and Discussion

Test Material Results

The test materials were first analyzed by OkaTest and LC/MS/MS to determine the content and profile of OA toxins. Results obtained by both methods for samples A, F, and G showed concentration for OA toxins below their LOD (44 and 40 µg/kg, respectively). The BM was tested by LC/MS/MS (19) at EURLMB, and no peaks were detected for this group of toxins (LOD for this method is 15 µg/kg). Therefore, materials A, F, G, and BM were considered blank; therefore, no homogeneity or stability studies were carried out.

Analyses by LC/MS/MS were used to identify the toxin profile and to ensure that all toxins belonging to the OA group were present in the materials. Table 2 shows concentration

Table 7. Details of the test materials, number of results submitted, and results after removing outliers, together with performance values of precision (repeatability and reproducibility) obtained for the colorimetric OkaTest[a]

							Repeatability[c]			Reproducibility[c]			
							µg total equiv.OA/kg						
Test material	Matrix	Runs/lab	No. labs submitting results	No. labs after invalid/incorrect results	No. of labs after outliers[b]	Mean (µg total equivalent OA/kg)[c]	S_r	r	RSD_r, %	S_R	R	RSD_R, %	HorRat
A	Mussel *M. galloprovincials*	2	16	14	—	<63	—	—	—	—	—	—	—
D	Clam *V. pullastra*	2	16	15	15 (0)	242	14.7	41.2	6.1	19.4	54.4	8.0	0.4
E	Mussel *M. galloprovincialis*	2	16	15	13 (2)	98.8 (102)	7.32 (20.8)	20.5 (58.4)	7.4 (20.5)	10.7 (19.6)	30.0 (54.8)	10.7 (19.2)	0.5 (0.8)
F	Scallop *P. maximus*	2	16	15	—	<63	—	—	—	—	—	—	—
G	Clam *V. decussatus*	2	16	14	—	<63	—	—	—	—	—	—	—
K	Clam *V. romboides*	2	16	15	14 (1)	275 (277)	14.9 (21.4)	41.8 60.1)	5.4 (7.7)	21.0 (25.0)	58.7 (70.1)	7.6 (9.0)	0.4 (0.5)
L	Cockle *C. edulis*	2	16	15	15 (0)	175	19.6	55.0	11.2	23.2	64.9	13.2	0.6
N	Mussel *M. edulis*	2	16	15	15 (0)	255	15.6	43.7	6.1	20.7	58.1	8.1	0.4

[a] S_r = Repeatability SD, S_R = reproducibility SD, RSD_r = repeatability RSD, RSD_R = reproducibility RSD, r = repeatability limit, R = reproducibility limit.

[b] Number of laboratories remaining after removal of outliers (number of outliers).

[c] Mean, repeatability, and reproducibility (values obtained including outliers).

in OA equivalents determined by OkaTest and toxins profile of the different materials used. All test materials were found to be stable for the duration of the study and with sufficient homogeneity (Tables 3 and 4).

Interlaboratory Study Results

All participants who received test materials reported results. The sample concentration was calculated by standard curves obtained by each laboratory every day of analysis. Fit parameters of each standard curve are shown Table 5. Although the slopes show differences depending on the laboratory and day, the calculated samples concentration was not affected. The data obtained by each laboratory per test material and day of analysis are shown in Table 6.

All individual values obtained per material, day and laboratory were also plotted. One graph per material is shown in Figure 1. The solid lines represent the assigned mean value obtained for each material in this study (Table 7). The area between the dashed lines demonstrates the range of deviation from the mean value based on the theoretical reproducibility SD ($PRSD_R$).

Two laboratories reported one of the assays with $R^2 < 0.96$; one (Laboratory A) repeated the analysis obtaining R^2 within the required criterion. Laboratory L, however, could not repeat the assay on time, and those results were considered invalid and removed for statistical analysis.

Materials A, F, and G were not statistically analyzed, as they were blank samples. However, Laboratory J reported values within the working range of the test for Materials A and G. These values are considered incorrect according to the AOAC guidelines (20), as they are positive values found for a blank material. All the other laboratories in the study identified the blank materials below the working range of the test.

The valid data from the contaminated test materials (D, E, K, L, and N) were then analyzed for identification of outliers applying Cochran and Grubbs tests (20). Results from Laboratory L could not be included in the statistical analysis, as only one value per material was available.

The Cochran test showed Laboratory G for Material K and Laboratory P for Material E as outliers. This test was applied again after these outliers were removed. Laboratory J for Material E was also excluded in a second round. The Grubbs single and pair values tests were then applied; no further outliers were identified.

The mean values assigned for OA-toxins for the test materials were 98.8, 175.4, 242.8, 255.0, and 275.0 µg total equivalents OA/kg for Materials E, L, D, N, and K, respectively (Table 7).

Values obtained for repeatability SD (S_r) ranged from 7.3 µg/kg for Material E to 19.6 µg/kg for Material L, with repeatability RSDs (RSD_r) from 5.4% for Material K to 11.2% for Material L (Table 7). The reproducibility SD (S_R) calculated for the five test materials ranged from 10.7 to 23.2 µg/kg, with reproducibility RSD (RSD_R) values from 7.6 to 13.2% for Materials K and L, respectively (Table 7).

The HorRat values obtained were 0.4 for Materials D, K, and N, 0.5 for Material E, and 0.6 for Material L (Table 7), indicating a very good performance of the method. These values are just at the lower limit of the range considered as normally expected for a good reproducibility of a method ($0.5 < HorRat \leq 1.5$), according to the AOAC guidelines (20). HorRat values between 0.64 and 2.61 for OA-toxins group (21), 0.3 and 2.0 for paralytic

Table 8. Results from the recovery experiment carried out during Day 2 of the interlaboratory study

	µg OA total eq./kg			
Lab code	BM[a]	Spiked concn.	BM + OA[b]	Recovery, %
A	—	161	172	107.1
B	—	161	162	100.7
C	—	161	155	96.3
D	—	161	115	71.6
E	—	161	124	77.3
F	—	161	138	85.5
G	—	161	162	100.7
H	—	161	131	81.1
I	—	161	152	94.4
J	—	161	197	122.3
K	—	161	152	94.4
L	—	161	196	121.6
M	—	161	153	95.0
N	—	161	174	108.3
O	—	161	155	96.3
P	—	161	185	114.7
Mean recovery, %				98.0
SD				14.2
RSD, %				14.5

[a] BM = Blank material. No OA toxins were detected; therefore, a concentration of zero was considered for calculation purposes.

[b] BM + OA = Concentration of the samples spiked with 161 µg/kg.

shellfish toxins (22) and 1.1 to 2.4 for domoic acid (23) were previously described for other methods.

The statistical analysis was also carried out including outliers (Table 7). Although there were some differences when including outlier values, repeatability and reproducibility remained satisfactory and within the expected values for this type of interlaboratory study.

Although the main objective of the validation study was to determine the repeatability and between-laboratory reproducibility of the OkaTest kit, a recovery assay was also carried out. A scallop blank sample (BM) was spiked with OA by each laboratory, and the recovery of OkaTest calculated. Recovery values from all participants ranged from 71.6 to 122.3%. The mean and RSD were 98.0 and 14.5%, respectively (Table 8). These recoveries met the criteria set in the AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals (24).

Comments from Participants

Most participants reported that the SOP for the method provided all the information they needed to perform the assay and that they did not have difficulties understanding any part of it. Some comments were made about the phosphatase preparation. Those led to the conclusion that the use of a nonorbital shaker does not always guarantee full dissolution of this reagent. Manual mixing, longer preparation, and a final visual check of the solution should be included in the SOP. Other minor comments were made, and were answered or resolved by the study director.

Conclusions

The precision and recovery values determined in this study for OkaTest can be considered satisfactory for this type of methodology and the concentration range required. The colorimetric PPIA, OkaTest, could be used as an assay complementary to the reference method for determination of the OA toxins group in molluscs according to the Commission Regulations (EC) No. 2074/2005 and No. 15/2011. Additional methods have to be implemented in a laboratory to analyze all regulated lipophilic marine biotoxins.

Acknowledgments

We thank the following collaborators for their participation, time, effort, and comments towards this study:

Dolores Calvo, ZEU-INMUNOTEC, Spain;

Monica Campàs, IRTA, Spain;

Guntis Cepurnieks, Institute of Food Safety, Animal Health and Environment, Latvia;

David Clarke, Marine Environment and Food Safety Services, Ireland;

Jorge Correa, INTECMAR, Spain;

Lorena A. Delgado, Public Health Institute, Chile;

Alejandra Goya A, SENASA, Argentina;

Katrin Kapp, BfR (Federal Institute for Risk Assesment), Germany;

Panagiota Katikou, Ministry of Rural Development and Food Centre of Veterinary Institutions of Thessaloniki Institute of Food Hygiene, Greece;

Mirslaw Michalski and Kataryna Graziel, National Veterinary Research Institute, Poland;

María José Chapela, ANFACO-CECOPESCA, Spain;

Sonia Piñero, European Reference Laboratory for Marine Toxins, Spain;

Vlad Serafim, The Institute for Diagnosis and Animal Health, Romania;

Ulrich Schwank, Bavaria's policies on health and consumer protection (LGL), Germany;

Andrew Turner and Clothilde Brunet, CEFAS, UK; and

Paulo Vale and Susana Rodrigues, IPIMAR, Portugal.

Note: Collaborative efforts among the European Reference Laboratory for Marine Toxins (EURLMB), NRLs, and ZEU-INMUNOTEC does not amount to an endorsement of the firm's products.

References

(1) Xing, Y., Xu, Y., Chen, Y., Jeffrey, P.D., Chao, Y., Lin, Z., Li, Z., Strack, S., Stock, J.B., & Shi, Y. (2006) *Cell* **127**, 341–353. http://dx.doi.org/10.1016/j.cell.2006.09.025

(2) Regulation (EC) No. 853/2004 (2004) *Off. J. Eur. Union* **L139**, 55–205

(3) Commission Regulation (EC) No. 15/2011 (2011) *Off. J. Eur. Union* **L6**, 3–6

(4) Commission Regulation (EC) No. 2074/2005 (2005) *Off. J. Eur. Union* **L338**, 27–59

(5) Takai, A., Bialojan, C., Troschka, M., & Rüegg, J.C. (1987)

FEBS Lett. **217**, 81–84. http://dx.doi.org/10.1016/0014-5793(87)81247-4

(6) Bialojan, C., & Takai, A. (1988) *Biochem. J.* **256**, 283–290

(7) Simon, J.F., & Vemoux, J.-P. (1994) *Nat. Toxins* **2**, 293–301. http://dx.doi.org/10.1002/nt.2620020508

(8) Honkanen, R.E., Stapleton, J.D., Bryan, D.E., & Abercrombie, J. (1996) *Toxicon* **34**, 1385–1392. http://dx.doi.org/10.1016/S0041-0101(96)00095-5

(9) Tubaro, A., Florio, C., Luxich, E., Sosa, S., Della Loggia, R., & Yasumoto, T. (1996) *Toxicon* **34**, 743–752. http://dx.doi.org/10.1016/0041-0101(96)00027-X

(10) Vieytes, M.R., Fontal, O.I., Leira, F., Baptista de Sousa, J.M.V., & Botana, L.M. (1997) *Anal. Biochem*. **248**, 258–264. http://dx.doi.org/10.1006/abio.1997.2127

(11) Mountfort, D.O., Kennedy, G., Garthwaite, I., Quilliam, M., Truman, P., & Hannah, D.J. (1999) *Toxicon* **37**, 909–922. http://dx.doi.org/10.1016/S0041-0101(98)00222-0

(12) Ramstad, H., Shen, J.L., Larsen, S., & Aune, T. (2001) *Toxicon* **39**, 1387–1391. http://dx.doi.org/10.1016/S0041-0101(01)00097-6

(13) Mountfort, D.O., Suzuki, T., & Truman, P. (2001) *Toxicon* **39**, 383–390. http://dx.doi.org/10.1016/S0041-0101(00)00144-6

(14) Gonzalez, J.C., Leira, F., Fontal, O.I., Vieytes, M.R., Arévalo, F.F., Vieites, J.M., Bermúdez-Puente, M., Muñiz, S., Salgado, C., Yasumoto, T., & Botana, L.M. (2002) *Anal. Chim. Acta* **466**, 233–246. http://dx.doi.org/10.1016/S0003-2670(02)00597-4

(15) Smienk, H.G.F., Calvo, D., Razquin, P., Domínguez, E., & Mata, L. (2012) *Toxins* **5**, 339–352. http://dx.doi.org/10.3390/toxins4050339

(16) Sassolas, A., Catanante, G., Hayat, A., & Marty, J.-L. (2011) *Anal. Chim. Acta* **702**, 262–268. http://dx.doi.org/10.1016/j.aca.2011.07.002

(17) Thompson, M., Ellison, S.L.R., & Wood, R. (2006) *Pure App. Chem.* **78**, 145–196. http://dx.doi.org/10.1351/pac200678010145

(18) Capela, M.J., Reboreda, A., Vieites, J.M., & Cabado, A.G. (2008) *J. Agric. Food Chem*. **56**, 8979–8986. http://dx.doi.org/10.1021/jf801572j

(19) Villar-González, A., Rodriguez-Velasco, M.L., & Gago, A. (2011) *J. AOAC Int*. **94**, 909–922

(20) *AOAC Official Methods of Analysis, Interlaboratory Collaborative Study* (2002) AOAC INTERNATIONAL, Gaithersburg, MD, Appendix D, p. 9

(21) Van den Top, H.J., Gerssen, A., & Van Egmond, H.P. (2011) *Report on Quantitative Determination of Liphophhilic Toxins in Shellfish by LC/MS/MS*, http://www.edepot.wur.nl/180890

(22) Van de Riet, J., Gibbs, R.S., Muggah, P.M., Rourke, W.A., & MacNeil, J.D. (2011) *J. AOAC Int*. **94**, 1154–1176

(23) Kleivdal, H., Kristiansen, S.I., Nilsen, M.V., & Gokoyr, V. (2007) *J. AOAC Int.* **90**, 1011–1027

(24) *AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals* (2002) AOAC INTERNATIONAL, Gaithersburg, MD

Toxins **2012**, *5*, 339-352; doi:10.3390/toxins4050339

OPEN ACCESS

toxins

ISSN 2072-6651
www.mdpi.com/journal/toxins

Article

Single Laboratory Validation of A Ready-to-Use Phosphatase Inhibition Assay for Detection of Okadaic Acid Toxins

Henry G. F. Smienk, Dolores Calvo, Pedro Razquin, Elena Domínguez * and Luis Mata

Zeu-Inmunotec, Polígono PLAZA, C/Bari 25 Dpdo, 50197, Zaragoza, Spain;
E-Mails: hsmienk@zeulab.com (H.G.F.S.); dcalvo@zeulab.com (D.C.);
prazquin@zeulab.com (P.R.); lmata@zeulab.com (L.M.)

* Author to whom correspondence should be addressed; E-Mail: edominguez@zeulab.com;
Tel.: +34-976731533; Fax: +34976524078.

*Received: 31 January 2012; in revised form: 4 April 2012 / Accepted: 18 April 2012 /
Published: 30 April 2012*

Abstract: A phosphatase inhibition assay for detection of okadaic acid (OA) toxins in shellfish, OkaTest, was single laboratory validated according to international recognized guidelines (AOAC, EURACHEM). Special emphasis was placed on the ruggedness of the method and stability of the components. All reagents were stable for more than 6 months and the method was highly robust under normal laboratory conditions. The limit of detection and quantification were 44 and 56 µg/kg, respectively; both below the European legal limit of 160 µg/kg. The repeatability was evaluated with 2 naturally contaminated samples. The relative standard deviation (RSD) calculated was 1.4% at a level of 276 µg/kg and 3.9% at 124 µg/kg. Intermediate precision was estimated by testing 10 different samples (mussel and scallop) on three different days and ranged between 2.4 and 9.5%. The IC_{50} values of the phosphatase used in this assay were determined for OA (1.2 nM), DTX-1 (1.6 nM) and DTX-2 (1.2 nM). The accuracy of the method was estimated by recovery testing for OA (mussel, 78–101%; king scallop, 98–114%), DTX-1 (king scallop, 79–102%) and DTX-2 (king scallop, 93%). Finally, the method was qualitatively compared to the mouse bioassay and LC-MS/MS.

Keywords: protein phosphatase inhibition assay (PPIA); protein phosphatase 2A (PP2A); validation; okadaic acid (OA); diarrheic shellfish poisoning (DSP)

1. Introduction

Diarrheic shellfish poisoning (DSP) is a consequence of the ingestion of a series of lipophilic toxins produced by dinoflagellates that can be present in shellfish for human consumption. These lipophilic toxins can be subdivided into four groups: the okadaic acid group (OA-toxins) including the dinophysistoxins (DTX), the pectenotoxin group (PTX), the yessotoxin group (YTX) and finally the azaspiracids (AZA). Only the OA-toxins and AZA are known to cause gastrointestinal problems [1,2]. For many years the mouse bioassay (MBA) has been the official method of detection for lipophilic toxins in the European Union [3], but with the publication of Commission Regulation (EU) No. 15/2011 [4], LC-MS/MS has become the reference method for their determination. This regulation also states that alternative or complementary methods can be used as long as an equivalent level of public health protection is provided, and the method performance criteria stipulated by the European Union Reference Laboratory on Marine Biotoxins (EU-RLMB) are fulfilled. Such methods should be intra-laboratory validated and successfully tested under a recognized proficiency test scheme.

Protein phosphatase inhibition assays (PPIA) have been identified for a long time as an alternative for the detection of OA-toxins, as ser/thr phosphatases are known to be their natural target [5,6]. As such, a validated phosphatase inhibition assay can be very useful in lipophilic toxin detection, complementary to the more complex, expensive and time consuming LC-MS/MS; or as an alternative when only OA-toxins are present in the samples. Different laboratories have developed in-house PPIA with good qualifications, using colorimetric or fluorimetric substrates to monitor enzyme inhibition. [7–12]. A collaborative study was also performed with a fluorimetric assay [13]. However, specific equipment, not often available in routine testing laboratories, makes difficult the use of fluorimetric assays for monitoring purposes. Besides, fluorimetric substrates are less stable than colorimetric ones and therefore less appropriate for ready-to-use kits. A standardized commercial test based on PPIA has not been available until recently. In this paper, we present a single laboratory validation of a commercial colorimetric PP2A assay (OkaTest) for the determination of OA-toxins in bivalve mollusks.

2. Materials and Methods

2.1. Reagents and Equipment

OkaTest kit (formerly Toxiline-DSP): The kit includes a 96-well microtiter plate, four vials of lyophilized protein phosphatase 2A (PP2A), purified from human red blood cells, five OA standards (0.5, 0.8, 1.2, 1.8 and 2.8 nM) prepared from the OA Certified Reference Material (NRC CRM-OA-c, NRC-CNRC, Institute for Marine Biosciences), a liquid chromogenic substrate (p-Nitrophenyl phosphate), phosphatase dilution buffer and buffer solution.

Other reagents not included in the OkaTest kit: Methanol (Reagent grade, Carlo Erba), HCl (Reagent grade, 37% v/v, Carlo Erba), NaOH (Reagent grade, Scharlau), de-ionized water (type II, ISO 3696), certified Reference Materials (NRC CRM-DSP-MUS-b, NRC CRM-OA-c, NRC-CNRC, Institute for Marine Biosciences), DTX-1 (042-28661, Wako) and DTX2 (00-DTX2, Cifga).

Equipment: Ultra homogenizer (IKA werken), a water bath at 76 ± 2 °C (Raypa), a FX-incubator at 30 °C ± 2 °C (ZEU-INMUNOTEC), a microplate absorbance reader (405 nm ± 10 nm wavelength

filter, Multiskan RC, Thermo-Labsystems), roller mixer, centrifuge, micropipettes, graduated 50 mL centrifuge tubes and laboratory glassware.

2.2. Sample Preparation

Market samples were thoroughly washed, the whole mollusk tissue recovered from the shell, and then blended. Portions of 5 ± 0.1 g were prepared and used for fresh testing, or stored frozen (below −15 °C) for future analysis. The portions were extracted by adding 25 mL of methanol (100% v/v) and mixing with a vortex for 2 min. The methanolic extract was separated by centrifugation for 10 min. at 2000 × g. To perform the hydrolysis, 640 µL of the methanolic extract and 100 µL of 3 N NaOH were mixed and incubated for 40 ± 1 min. at 76 ± 1 °C. To stop the reaction, 80 µL of HCl were added and sample preparation buffer used to make up a final volume of 20 mL. For non-hydrolyzed samples, 640 µL of methanolic extract were diluted up to 20 mL with sample preparation buffer. Hydrolysis was carried out in most samples unless otherwise specified.

2.3. Assay Procedure

The phosphatase solution was prepared by adding 2 mL of dilution buffer to each vial of lyophilized PP2A. To assure full hydration of the lyophilized enzyme, it was mixed gently for 1 h ± 5 min. at room temperature (22 °C ± 2 °C) on a roller mixer. Then, 50 µL of samples or ready-to-use OA standards (0.5, 0.8, 1.2, 1.8 and 2.8 nM), and 70 µL of the prepared phosphatase solution were added in duplicate to a microwell plate. This mixture was equilibrated in an incubator for 20 ± 2 min. at 30 °C. Finally, 90 µL of the chromogenic substrate were added to each well and incubated for 30 ± 2 min. at 30 °C. The absorbance was read at 405 nm.

2.4. Calculations

The results were calculated from a standard curve by plotting the absorbance values in a linear *y axis* and the concentration of OA in a logarithmic *x axis*, and using a logarithmic fitting. As an acceptability criterion for the assay, the Pearson correlation coefficient r^2 had to be greater than or equal to 0.96. The OA concentration contained in the sample was then calculated using the following equation:

$$x = \mathrm{EXP}\ (y - \mathrm{b})/\mathrm{a}$$

where x is the OA concentration in the sample (C_s) and y the absorbance of the sample.

The OA-toxin concentration in shellfish tissue was calculated as follows:

$$C_t\ (\mu g/kg) = (C_s\ (nM) \times FD \times MW\ (g/mol) \times V_e\ (L))/M_t\ (g)$$

where C_t is the toxin concentration in tissue, expressed as equivalents of OA, *FD* is the methanolic extract dilution factor (31.25), *MW* is the OA molecular weight = 805, V_e is the methanolic extract volume (0.025 L), M_t is the tissue weight (5 g).

Samples with an OA concentration falling outside the working range (<0.5 nM or >2.8 nM) will be reported as <63 µg/kg (or <0.5 nM) or >352 µg/kg (or >2.8 nM), respectively.

2.5. Ruggedness Testing

The ruggedness testing was performed by introducing changes in the procedure and determining the effects on the sample quantification [14]. The variations used were chosen according to the values expected under normal laboratory conditions.

2.6. Spiking Procedure

Samples were spiked with OA Certified Reference Calibration Solution (NRC CRM-OA-c). The reference solution was prediluted to 2 µM in sample buffer and added accordingly. No Certified Reference Materials were available for DTX-1 and DTX-2 at the time of the performance testing. These toxins were first dissolved in methanol and diluted to 2 µM in sample buffer before adding to the samples.

A Certified Reference Material (NRC CRM-DSP-MUS-b) was also tested. However, the certified concentration of this material is far above the working range of the assay and the sample had to be diluted with blank mussel or king scallop. To do this, an amount of reference material was added as precisely as possible to 50 mL tubes, and weighed. The blank material was added on top and the mixture weighed again. Then, the amount of the mussel reference material per sample was calculated. This value was used as the theoretical spiked amount. The samples were analyzed with and without hydrolysis, as the reference material was only certified for OA and DTX-1, but ester derivates of the OA-toxins could also be present as indicated in the CRM certificate. The total recovery was calculated according to the AOAC Official methods of analysis [15].

2.7. Method Comparison

A method comparison was also carried out with OkaTest, the mouse bioassay (MBA) and LC-MS/MS, using EU harmonized protocols for the last two methods [16,17].

Shellfish samples were previously tested by a third party laboratory using mouse bioassay (MBA) and LC-MS/MS, and kindly donated to do the method comparison.

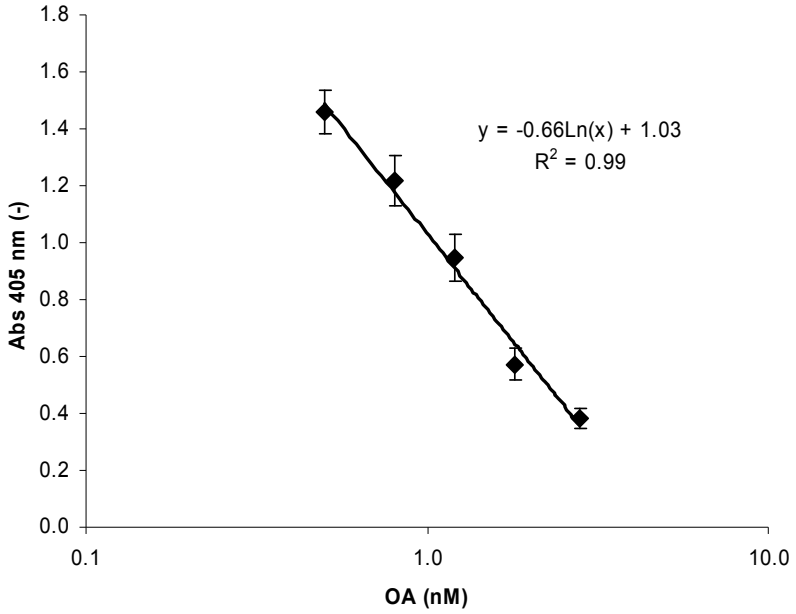
As MBA is a qualitative method, results obtained by OkaTest and LC-MS/MS were interpreted qualitatively for comparison purposes. Therefore, samples with a concentration ≥160 µg/kg were regarded as positive, while samples with a concentration <160 µg/kg were reported negative.

3. Results and Discussion

3.1. Calibration of the Assay

The assay is calibrated by five OA standards prepared by dilution from the NRC CRM-OA-c with a concentration between 0.5 and 2.8 nM OA. Following the kits sample preparation (see material and methods), this will result in a working range between 63 and 352 µg/kg. Figure 1 shows a typical calibration curve from 5 different assays using different phosphatase batches. All calibration curves were evaluated according to the Pearson correlation coefficient obtained after a logarithmic fitting procedure ($r^2 > 0.96$).

Figure 1. Typical calibration curve of OkaTest produced as the mean of 5 phosphatase batches. The Pearson correlation coefficient (r^2) of the logarithmic fit was >0.96 for each batch. The figure shows the equation and r^2 of the mean. The error bars were calculated as ±1 SD.



The bias introduced by the logarithmic fitting procedure on the calibration curve of the kit was estimated by recalculating the concentration of the OA dilutions using its own standard curve. The relative absolute difference was then calculated as the absolute difference between the theoretical and calculated OA concentration divided by the theoretical OA concentration and multiplied by 100 (Table 1). The best accuracy was found at levels around the regulatory limit (0.8% at 1.2 nM OA standards equals 151 µg OA equivalents/kg mollusk), while below that level (0.5 nM of OA), a 9.0% overestimation was calculated. Only minor deviations were calculated over the legal limit.

Table 1. Bias introduced due to the fitting procedure. Relative absolute difference was calculated from mean of 5 standard curves by relating the absolute difference to the theoretical OA concentration.

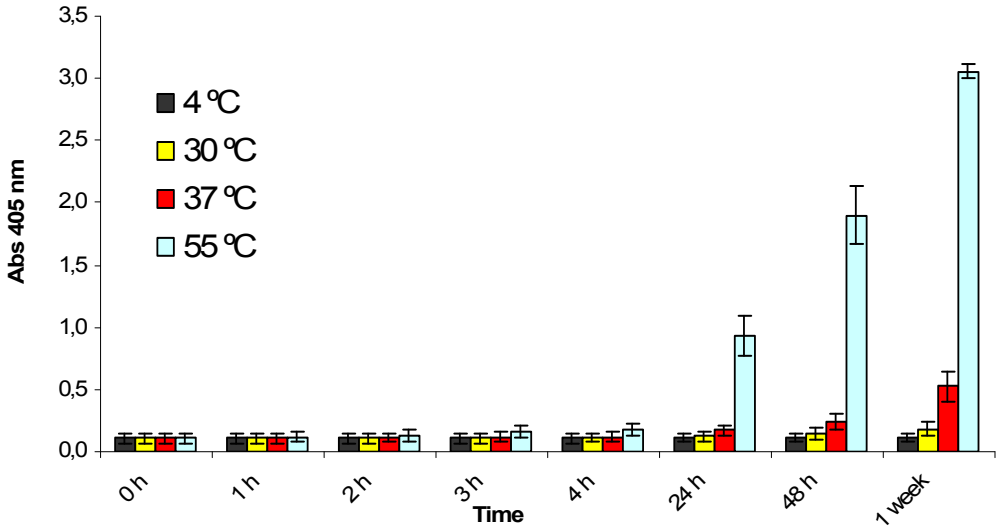
OA theoretical (nM)	OA calculated (nM)	Relative Absolute Difference
0.50	0.55	9.0%
0.80	0.83	3.8%
1.20	1.21	0.8%
1.80	1.78	1.1%
2.80	2.73	2.5%

3.2. Stability and Homogeneity of the Components

The stability and homogeneity of the critical components of the kit were studied by combining a real time and accelerated study design. Water soluble buffers such as the phosphatase dilution solution and the sample buffer were considered less critical, as sufficient internal know-how was available for these components and no stability problems were expected. Other components, such as the

ready-to-use chromogenic substrate, the PP2A or the OA standards, were specially developed for the phosphatase inhibition assay and were more extensively tested. Reagents were normally analyzed within the assay system or by performing specific tests depending on their particular characteristics. The ready-to-use substrate performed correctly in the OkaTest assay when stored for a year at temperatures between 2 and 15 °C (results not shown), as the background absorbance remained acceptable (below 0.3 absorbance units). However, accelerated studies showed that the substrate is sensitive to higher temperatures (Figure 2). After 24 h at 55 °C, the substrate was strongly hydrolyzed and after 1 week at 37 °C the absorbance of the substrate was above 0.6. Nevertheless, these results indicate that although the hydrolysis rate increases with temperature, it is very stable at temperatures below 15 °C and no problems should be expected under normal conditions of usage and storage.

Figure 2. Study of the temperature stability for the ready-to-use chromogenic substrate (p-Nitrophenyl phosphate). Absorbance at 405 nm was measured at different times and temperatures. Assays were performed in triplicate. The error bars were calculated as ±1 SD.



The OA standards and the PP2A were estimated the most critical components, as their quantity and quality establish the working range and, to a great extent, the ruggedness of the assay. The enzyme quantity determines the amount of analyte that is needed for inhibition, while the enzyme quality assures the amount of product formed per time unit [18]. Likewise, the lack of stability or impurities of the OA standards directly affect the quantification, either overestimating, in the case of degradation of the OA, or underestimating, when impurities that can inhibit the PP2A are present. Therefore, greater emphasis was put on these components and the 'between batch homogeneity' was evaluated besides the stability of the components. The between batch homogeneity was studied by taking 1 set of standards or 1 vial of phosphatase from 5 different batches. These batches were chosen along the estimated shelf life of the compounds and tested in one single assay together with internal control samples. All batches performed according to the assays' specifications ($r^2 > 0.96$) and the relative standard deviation was far below 15%, the expected value for samples assayed under repeatability conditions [19]. These results proved the stability of the enzyme for over 12 months at 4 °C and the homogeneity of between all batches tested (Table 2).

Table 2. Phosphatase stability and homogeneity. Five different phosphatase batches were tested at different stages of shelf life. Mean, standard deviation (SD) and relative standard deviation (RSDr) were calculated. Three internal control samples were used to verify correct quantification.

PP2A batch (shelf life)	Sample 1 (µg/kg)	Sample 2 (µg/kg)	Sample 3 (µg/kg)
1 (2 months)	95	160	310
2 (4 months)	100	169	304
3 (8 months)	88	162	323
4 (10 months)	94	156	300
5 (12 months)	90	144	341
mean	93	158	316
SD	5	9	17
RSDR	4.8%	6.0%	5.2%

For the OA standards, the same strategy was used. Five batches, covering 90% of the shelf life of the component (6 months), were tested in one assay to be able to single out the variation due to the standards' stability and homogeneity (Table 3). A sample shown to be blank (0 nM) was included to be able to calculate the effect of variables other than OA. The RSDr calculated from the absorbance values were all <3%, proving the stability and homogeneity of the standards over 6 months.

Table 3. OA standards stability and homogeneity. Five different batches of OA standards were tested at different stages of shelf life. The absorbances (405 nm) obtained for each of the standards are shown. Mean, standard deviation (SD) and relative standard deviation (RSDr) of these absorbances were calculated.

	Absorbance 405 nm							
Standards OA (nM)	batch 1 5 months	batch 2 4 months	batch 3 3 months	batch 4 2 months	batch 5 1 week	mean	SD	RSDr
0.0	2.042	2.100	2.064	2.073	2.120	2.079	0.031	1.5%
0.5	1.622	1.614	1.649	1.625	1.678	1.637	0.026	1.6%
0.8	1.462	1.390	1.386	1.375	1.372	1.397	0.037	2.7%
1.2	1.124	1.116	1.101	1.092	1.134	1.113	0.017	1.5%
1.8	0.772	0.792	0.769	0.822	0.809	0.793	0.023	2.9%
2.8	0.619	0.646	0.606	0.637	0.613	0.624	0.017	2.7%

3.3. Ruggedness

Enzymatic assays, such as OkaTest, can be sensitive to environmental factors, such as temperature, incubation time or reagent volume. To determine the impact of these factors, samples with concentrations around the regulatory limit were quantified at normal and suboptimal conditions (Table 4). The effect of temperature was tested by performing the OkaTest assay at three different temperatures 28, 30 and 32 °C, obtaining a RSD of 1.0%. These results showed that temperature variations of 2 °C did not affect the performance as RSDr values were lower than 10% usually obtained in the assay (Table 5).

Duration and pipetting volumes were evaluated alike and none of the variables affected the results of the test, with the exception of large pipetting errors. Pipetting errors of 5 µL in samples or phosphatase addition (errors of 10% and 7.1%, respectively) gave RSDr values of 14% and 17%, respectively. Precision in substrate addition was less critical. Pipetting samples and phosphatase are, however, the main sources of variability affecting PPIA and therefore care should be taken when adding these components.

Table 4. Ruggedness testing. The effects of variations of the normal assay conditions on sample quantification are shown.

Variable	Normal value	Variation	Mean value (µg/kg)	RSDr
Temperature	30 °C	±2 °C	175	1.0%
Pre-incubation	20 min	18, 20, 22, 24 min	158	3.6%
Incubation	30 min	27, 30, 33, 36 min	147	2.9%
Syst. pipetting error	50, 70, 90 µL	±2 µL	155	4.3%
Random pipetting error				
Sample	50 µL	±5 µL	151	14%
PP2A	70 µL	±5 µL	153	17%
Substrate	90 µL	±5 µL	158	6.1%
Phosphatase solubility time	60 ± 5 min	±30 min	158	5.0%

Table 5. Intermediate precision of ten different mussel and scallops samples. Mean, standard deviation (SD), relative standard deviation (RSDr) were calculated. < 63: below the working range of the assay (63–352 µg/kg).

Sample	Origin	Day 1 (µg/kg)	Day 2 (µg/kg)	Day 3 (µg/kg)	Mean	SD	RSDr
1	Mussel	211	227	187	208	20	9.5%
2	Mussel	122	132	113	122	10	7.8%
3	Scallop	<63	<63	<63	-	-	-
4	Mussel	82	94	90	88	6	7.0%
5	Mussel	196	196	215	202	11	5.2%
6	Scallop	<63	<63	<63	-	-	-
7	Mussel	<63	<63	<63	-	-	-
8	Scallop	125	108	117	117	8	7.0%
9	Mussel	250	253	281	261	17	6.5%
10	Mussel	277	279	289	282	7	2.4%

3.4. Applicability

There are numerous descriptions of the application of protein phosphatase inhibition assays for determination of OA and its derivatives [7–13]. However, the inhibition pattern of OA, DTX1 and DTX2 is different and is supposed to correspond to their toxicity. One way to evaluate the inhibition capacity of toxins on an enzyme is by determining the IC_{50}, the concentration of toxin able to inhibit 50% of the maximum enzyme activity. This concentration depends, among others, on the amount of enzyme and the substrate concentration present in the assay [20] and therefore the IC_{50} values published for these toxins are difficult to compare [7,8,12,18,21,22]. The IC_{50} values found in our study were 1.2 nM for both OA and DTX-2, and 1.6 nM for DTX-1 (Figure 3) and are in accordance

with the ones obtained recently by Huhn *et al*., 2009 [21]. However, these do not exactly correspond to the toxicity factors (TEF) that are used in analytical methods such as LC-MS/MS; as OA and DTX-1 have a TEF of 1, while DTX-2 has a TEF of 0.6, indicating equal toxicity for DTX-1 and OA and less toxicity for DTX-2 [2]. According to these values, our results would lead to an overestimation of the amount of DTX-2 and an underestimation of the amount of DTX-1 when compared with methods such as LC-MS/MS. However, the recovery data obtained for both DTX-1 and DTX-2 were similar to the ones obtained for OA (Table 6) suggesting that difference has a low impact in the determination of the level of toxins in shellfish samples.

Figure 3. Phosphatase inhibition curve obtained with okadaic acid (OA), dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2). Each point is the mean obtained from three different phosphatase batches. The standard deviation is not shown to maintain the figure legible. The IC_{50} values were 1.2 nM for both OA and DTX-2, and 1.6 nM for DTX-1.

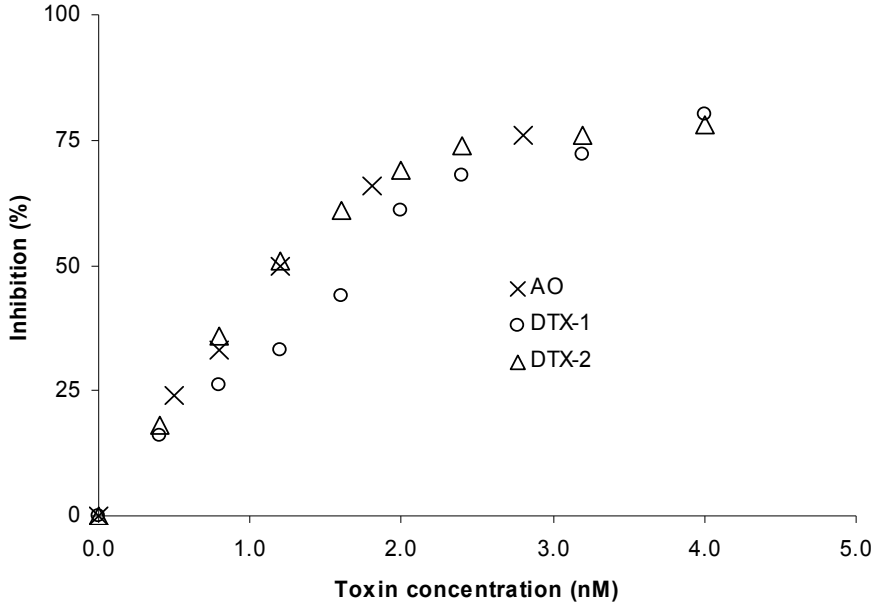


Table 6. Recovery of the different toxins was calculated testing 5 samples at 0.5, 1 and 1.5 times the regulatory limit on 3 different days. OA Certified Reference Material (NRC CRM-OA-c) was spiked on mussel and king scallop. DTX-1 and DTX-2 were spiked on king scallop. ND: not determined.

Toxin	Matrix	Recovery (RSDr)		
		80 µg/Kg	160 µg/Kg	240 µg/Kg
OA	Mussel	101% (15%)	90% (8.9%)	78% (5.4%)
	King scallop	114% (9.9%)	98% (8.4%)	106% (8.7%)
DTX-1	King scallop	102% (15%)	79% (12%)	88% (17%)
DTX-2	King scallop	93% (2.3%)	ND	ND

3.5. Limit of Detection, Limit of Quantification, Repeatability and Reproducibility

The limit of detection (LOD) and limit of quantification (LOQ) were determined by using a blank +3 SD or blank +10 SD approach [14]. For blank mussel material, the LOD and LOQ were 44 and

56 µg/kg, respectively. These values are both below the working range of the test and sufficiently below the current European legal limit of 160 µg/kg.

To estimate the precision, the assay was tested both under repeatability and intermediate precision conditions. The repeatability characteristics were estimated by analyzing 8 fractions of two naturally contaminated mussel samples and RSDr of 1.4% with a mean of 276 µg/kg, and 3.9% with a mean of 124 µg/kg were obtained (results not shown). The intermediate precision of the test was estimated by analyzing 7 samples with OA-toxin levels covering the working range of the assay on three different days by the same analyst. For all samples, the RSDr was well below the 15% RSDr limit as calculated by Horwitz [19]. Three samples tested as negative by LC-MS/MS were included to evaluate the consistency of the negative results (Table 5).

3.6. Accuracy

The accuracy of the method was estimated by calculating recoveries for OA, DTX-1 and DTX-2 and by testing a Certified Reference Material (NRC-CNRC). Five portions containing 5 grams of mussel or king scallop were spiked with one of the three toxins at 0.5, 1 and 1.5 times the regulatory limit (80, 160 and 240 µg/kg), except for DTX-2 that was only added up to a concentration of 80 µg/kg. The five portions were analysed on three different days to determine the intermediate precision characteristics of the test. OA recoveries between 78 and 101% in mussel and 98 and 114% in king scallop were obtained. RSDr values for this toxin were below or equal to 15%. Similar recoveries were obtained for the other two toxins (Table 6). These recoveries are in agreement with the 75 to 120% range that is expected for this concentration range [19]. The RSDr results in this study were higher than the ones obtained in the precision experiments (Table 4), specially for DTX-1. This might be a consequence of the spiking. As mentioned before, the higher IC_{50} for DTX-1 compared to OA and DTX-2 had a low impact on the recovery.

Finally, four aliquotes of blank samples were spiked with the Certified Reference Material. The methanolic extract obtained was analysed with and without hydrolysis, and the recovery was estimated using the DTX-1 and OA content reported for the certified material. The recovery for the non-hydrolysed samples ranged from 71% to 98%, with a mean of 87% for mussle and 91% for king scallop (Table 7). These are acceptable recoveries and in accordance with the results showed in Table 6. However, the mean recovery of the hydrolysed samples was a 146% and 163% for mussle and king scallop, respectively. These percentages were far above the expected content of OA-toxins indicated in the reference material [23]. This could be due to the fact that the material is only certified for OA and DTX-1. Other esters of OA and DTX are reported in the certificate of anlaysis for this material.

Table 7. Recovery experiment with Certified Reference Material (NRC CRM-DSP-MUS-b). Samples were analysed with and without hydrolysis.

Matrix	Spiked level (µg/kg) (*n*)	Without hydrolysis		With hydrolysis	
		Recovery	RSDr	Recovery	RSDr
mussel	219 (4)	87%	14%	146%	12%
king scallop	180 (4)	91%	5.0%	163%	2.8%

3.7. Method Comparison

A method comparison among MBA, LC-MS/MS and OkaTest was performed with a total of 37 samples. Results were compared qualitatively for all three methods and quantitatively between OkaTest and LC-MS/MS. The 160 µg/kg regulatory limit was used to decide whether the samples were positive or negative (Table 8).

Table 8. Methods comparison. Results from OkaTest, MBA and LC-MS/MS. 31 of the 37 samples were tested by MBA. Positive results (+): ≥160 µk/kg. Negative results (−): <160 µg/kg. LOQ. Limit of quantification. NA: not available.

ID	M	MBA	LC-MS/MS	OKATEST	LC-MS/MS	OKATEST
1	**Cockle**	-	-	-	<LOQ	<LOQ
2	**Cockle**	+	+	+	193	252
3	**Donax**	-	-	-	82	97
4	**Mussel**	+	+	+	502	232
5	**Mussel**	+	-	+	<LOQ	268
6	**Mussel**	+	+	+	604	>352
7	**Mussel**	+	+	+	894	>352
8	**Mussel**	+	+	+	414	306
9	**Mussel**	+	+	+	444	>352
10	**Mussel**	NA	-	-	<LOQ	<LOQ
11	**Mussel**	NA	+	+	357	>352
12	**Mussel**	NA	-	-	<LOQ	<LOQ
13	**Mussel**	NA	-	-	<LOQ	<LOQ
14	**Mussel**	-	-	-	<LOQ	122
15	**Mussel**	+	-	+	158	196
16	**Mussel**	+	+	+	177	250
17	**Mussel**	+	+	+	288	265
18	**Mussel**	+	+	+	202	196
19	**Mussel**	+	+	+	390	277
20	**Mussel**	+	+	+	658	305
21	**Mussel**	+	+	+	392	310
22	**Mussel**	+	+	+	329	315
23	**Mussel**	+	+	+	232	270
24	**Mussel**	+	+	+	235	277
25	**Mussel**	+	-	-	152	135
26	**Mussel**	+	-	+	98	164
27	**Mussel**	+	+	+	168	211
28	**Mussel**	+	+	+	209	251
29	**Mussel**	+	-	+	113	191
30	**Mussel**	NA	+	-	292	<LOQ
31	**Mussel**	NA	+	+	316	304
32	**Mussel**	-	-	-	<LOQ	<LOQ
33	**Mussel**	+	+	-	177	124
34	**Mussel**	+	+	+	247	216
35	**Mussel**	+	+	-	185	144
36	**Scallop**	+	+	+	184	264
37	**Scallop**	-	-	-	<LOQ	<LOQ

In general, the qualitative interpretation of the results indicates that the three methods obtained equivalent results, especially taking into account that these are conceptually different methods. The OkaTest disagreed with both MBA and LC-MS/MS on two occasions (samples 33 and 35). OkaTest detected levels of OA-toxins in those two samples, but below the EU regulatory limit (124 and 144 µg/kg), while the samples were positive according to the other two methods). A third sample (25) was also identified as negative by OkaTest and positive by MBA. LC-MS/MS also gave a negative result for sample 25. The concentration of this sample determined by both methods was just below the EU regulatory limit.

The LC-MS/MS differed on four occasions: all four negative according to LC-MS/MS, but positive by the other two methods. Three of the samples (15, 26 and 29) contained OA-toxins below the EU refulatory limit, but sample 5 was quantified under the method's LOQ. Finally, one sample (30) was positive by LC-MS/MS, but under the LOQ by OkaTest. Sample 30 was not tested by MBA due to lack of material.

Quantitative results obtained by LC-MS/MS and Okatest showed some differencies. About two thirds of the samples gave similar results (±25%) with both methods, but the rest of the samples did not show a clear tendency. There is no evident explanation for this and further investigation would be required.

4. Conclusions

A colorimetric phosphatase inhibition assay for determination of OA-toxins, OkaTest, was single laboratory validated according to international methods validation guidelines. The limit of quantification of the method is well below the EU regulatory limit and the method permitted the easy quantification of up to 43 samples within one hour, excluding sample preparation. The method is robust, with very good precision characteristics, adequate specificity and accuracy.

This colorimetric phosphatase inhibition assay could be used as a complementary assay to the reference method for determination of lipophilic toxins, once a collaborative study has been completed and it has been successfully tested under recognized proficiency tests. This assay could be applied for monitoring purposes when OA-toxins are identified to be responsible for a bloom.

Acknowledgements

We thank María Rodríguez Velasco, from the EURLMB (European Reference Laboratory for Marine Biotoxins), for revising the data, providing the mollusk material and coordinating the method comparison. We also want to thank Adriano Rodriguez from the EURLMB for performing the LC-MS/MS determination and Anfaco-Cecopesca for the MBA and mollusk material.

Note: Collaborative efforts between the EURLMB and ZEU-INMUNOTEC do not amount to an endorsement of the firm's products.

References

1. Gerssen, A.; Pol-Hofstad, I.E.; Poelman, M.; Mulder, P.P.J.; van Den Top, H.J.; de Boer, J. Marine Toxins: Chemistry, toxicity, occurrence and detection with special reference to the Dutch situation. *Toxins* **2010**, *2*, 878–904.

2. EFSA. Marine biotoxins in shellfish-okadaic acid and analogues—Scientific Opinion of the Panel on Contaminants in the Food Chain. *EFSA J*. **2008**, *589*, 1–62.
3. European Commission. EC Commission Regulation (EC) No. 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No. 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No. 854/2004 of the European Parliament and of the Council and Regulation (EC) No. 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No. 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No. 854/2004. *Off. J. Eur. Comm.* **2005**, *338*, 27–59.
4. European Commission. EC Commission Regulation (EC) No. 15/2011 of 10 January 2011 amending Regulation (EC) No. 274/2005 as regards recognized testing methods for detecting marine biotoxins in live bivalve molluscs. *Off. J. Eur. Comm.* **2011**, *6*, 3–6.
5. Takai, A.; Bialojan, C.; Troschka, M.; Rüegg, J.C. Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin. *FEBS Lett*. **1987**, 21781–21784.
6. Bialojan, C.; Takai, A. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. *Biochem. J*. **1988**, *256*, 283–290.
7. Tubaro, A.; Florio, C.; Luxich, E.; Sosa, S.; Della Loggia, R.; Yasumoto, T. A protein phasphatase 2A inbhition assay for a fast and sensitive assessment of okadaic acid contamination in mussels. *Toxicon* **1996**, *34*, 743–752.
8. Honkanen, R.E.; Stapleton, J.D.; Bryan, D.E.; Abercrombie, J. Development of a protein phosphatase-based assay for the detection of phosphatase inhibitors in crude whole cell and animal extracts. *Toxicon* **1996**, *34*, 1385–1392.
9. Mountfort, D.O.; Kennedy, G.; Garthwaite, I.; Quilliam, M.; Truman, P.; Hannah, D.J. Evaluation of the fluorimetric protein phosphatase inhibition assay in the determination of okadaic acid in mussels. *Toxicon* **1999**, *37*, 909–922.
10. Ramstad, H.; Shen, J.L.; Larsen, S.; Aune, T. The validity of two HPLC methods and a colorimetric PP2A assay related to the mouse bioassay in quantification of diarrheic toxins in blue mussels (*mytilus edulis*). *Toxicon* **2001**, *39*, 1387–1391.
11. Simon, J.F.; Vemoux, J.P. Highly sensitive assay of okadaic acid using protein phosphatase and paranitrophenyl phosphate. *Nat. Toxins* **1994**, *2*, 293–301.
12. Vieytes, M.R.; Fontal, O.I.; Leira, F.; Baptista de Sousa, J.M.V.; Botana, L.M. A fluorescent microplate assay for diarrheic shellfish toxins. *Anal. Biochem*. **1997**, *248*, 258–264.
13. Gonzalez, J.C.; Leira, F.; fontal, O.I.; Vieytes, M.R.; Arévalo, F.F.; Vieites, J.M.; Bermúdez-Puente, M.; Muñiz, S.; Salgado, C.; Yasumoto, T.; *et al*. Inter-laboratory validation of the fluorescent protein phosphatase inhibitions assay to determine diarrheic shellfish toxins: Intercomparison with liquid chromatography and mouse bioassay. *Anal. Chim. Acta* **2002**, *466*, 233–246.
14. EURACHEM. *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*; EURACHEM: Prague, Czech Republic, 1998.
15. AOAC. *Appendix D: Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis*; AOAC International: Gaithersburg, MD, USA, 2002.

16. EURLMB. EU-Harmonised Standard Operating Procedure for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS. In *EU Harmonised SOP LIPO LCMSM Version 1*; EURLMB: Vigo, Spain, 2006;
17. EURLMB. EU Harmonised Standard Operating Procedure for detection of lipophilic toxins by mouse bioassay. In *EU Harmonised SOP MBA Lipophilic Version 4*; EURLMB: Vigo, Spain, 2007;
18. Takai, A.; Murata, M.; Torigoe, K.; Isobe, M.; Mieskes, G.; Yasumoto, T. Inhibitory effect of okadaic acid derivatives on protein phosphatases. *Biochem. J*. **1992**, *284*, 539–544.
19. Horwitz, W. *AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals*; AOAC International: Gaithersburg, MD, USA, 2002; pp. 12–19.
20. Engel, P.C. *Enzymology Labfax*; Academic Press: San Diego, CA, USA, 1996.
21. Huhn, J.; Jeffrey, P.D.; Larsen, K.; Rundberget, T.; Rise, F.; Cox, N.R.; Arcus, V.; Shi, Y.; Miles, C.O. A structural basis for the reduced toxicity of dinophysistoxin-2. *Chem. Res. Toxicol*. **2009**, *22*, 1782–1786.
22. Aune, T.; Larsen, S.; Aasen, J.A.B.; Rehman, N.; Satake, M.; Hess, P. Relative toxicity of dinphysistoxin-2 (DTX-2) compared with okadaic acid, based on acute intraperitoneal toxicity in mice. *Toxicon* **2007**, *49*, 1–7.
23. Perez, R.A.; Crain, S.M.; Walter, J.A.; Quilliam, M.A.; Melanson; J.E. *NRC CRM-OA-c*, *Certified Calibration Solution for Okadaic Acid*; Technical Report for CRMP: Halifax, Canada, 2008.

Collaborative study to validate a colorimetric phosphatase inhibition assay for determination of OA-toxins group in molluscs: TOXILINE-DSP.Co

Report

Coordination:

ZEU-INMUNOTEC S.L.
Polígono Plaza, C/ Bari 25, dpdo
50197 Zaragoza, Spain
e-mail: edominguez@zeulab.com

ANFACO CECOPESCA
Ctra. Colexio Universitario, 16
Lagoas (Marcosende)
36310 Vigo (Pontevedra)
e-mail: agcabado@anfaco.es

Validation Management Team (VMT):

- María L. Rodríguez-Velasco, EURLMB-AESAN
- David Clarke, NRL-Ireland
- Katrin Kapp, NRL- Germany
- Panagiota Katikou, NRL-Greece
- Elena Domínguez, ZEU-INMUNOTEC

Date of Circulation: 23.01.11
File Identifier: Validation Report. TOXILINE-DSP.Co, G-COM-OA 10. Rev. 1

TABLE OF CONTENTS

ABSTRACT

A collaborative study to validate a colorimetric phosphatase inhibition assay for determination of the Okadaic Acid toxins group in molluscs, TOXILINE-DSP.Co, was conducted.

A total of 16 test materials (8 materials in blind duplicates), including mussels, scallops, clams and cockles were analysed to determine the assay repeatability and between laboratory reproducibility. A recovery assay was also performed to evaluate the accuracy of the methodology.

The study was carried out by a total of 16 laboratories, from 11 different countries (9 European and 2 American).

Samples were extracted with 100% methanol and centrifuged. Then, samples were hydrolysed with NaOH, at 76ºC for 40 minutes, to be able to determine the total OA toxin content, including the esterified forms. The diluted hydrolysed extract was then analysed by TOXILINE DSP.Co test, where it was first incubated with a phosphatase enzyme at 30ºC for 20 min, and then with a colorimetric substrate at 30ºC for 30 min. Five Okadaic Acid (OA) standards were included in the assay obtaining a standard curve to calculate the concentration of OA-toxins group.

The overall mean values assigned for OA-toxins group for the test materials were 98.8, 175.4, 242.8, 255.0 and 275.0 µg total equivalents OA/kg.

Values obtained for repeatability standard deviation (Sr) ranged from 7.3 to 19.6 µg/Kg, with repeatability relative standard deviations (RSDr) between 5.4% and 11.2% (mean 7.5%).

The estimated reproducibility standard deviation (S_R) was from 10.7 to 23.2 µg/Kg, with reproducibility relative standard deviation (RSDR) values between 7.6 % and 13.2 % (mean 9.9%).

The HORRAT values, the ratio between RSDR and a theoretically calculated RSDR, obtained were between 0.4 and 0.6.

During the accuracy assay a mean recovery of 98.0% was obtained, with a relative standard deviation (RSD) of 14.5%.

The results obtained in this validation study indicate that the colorimetric phosphatase inhibition assay, TOXILINE-DSP.Co, is suitable for determination of the OA-toxins group. TOXILINE-DSP.Co could be used as an alternative or complementary test to the reference method for monitoring the OA-toxin group.

1. INTRODUCTION

Okadaid acid (OA) and its analogues, DTX1, DTX2, together with their ester forms are known as the group of OA-toxins. These toxins, lipophilic and heat stable, are produced by dinoflagellates and can be found in various species of shellfish, mainly in filter feeding bivalve molluscs.

The OA-toxins group causes Diarrhoeic Shellfish Poisoning (DSP), which is characterised by symptoms such as diarrhoea, nausea, vomiting and abdominal pain. These symptoms may occur in humans shortly after consumption of contaminated bivalve molluscs such as mussels, clams, scallops or oysters. Inhibition of serine/threonine phosphoprotein phosphatases is assumed to be responsible for these toxic effects [1]. These compounds are also involved in tumour promotion [2].

Regulation (EC) No 853/2004 [3] states that live bivalve molluscs placed on the market for human consumption must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed 160 µg of okadaic acid equivalents per kilogram for okadaic acid, dinophysistoxins and pectenotoxins together.

Commission Regulation (EU) No. 15/2011 [4], that amends Regulation No. 2074/2005 [5]; specifies the recognised testing methods for marine biotoxins. This regulation has just been published and will be applied from July 2011.

Regulation No 15/2011 indicates that in the case of lipophilic toxins and, among them OA-toxins, liquid chromatography-mass spectrometry technique (LC-MS/MS) is the reference method for routine testing of official controls or any checks done by food operators. Biological methods (mouse and rat bioassay) considered the reference ones by the Regulation 2074/2005, will only be used for a limited period of time.

Both regulations also considered other methods for routine testing of lipophilic toxins, providing they are intra-laboratory validated and successfully tested under a recognised testing scheme. Moreover, the phosphatase inhibition assay is specifically mentioned in these Regulations as an alternative or complementary method.

The TOXILINE-DSP test kit was developed by ZEU- INMUNOTEC and based on the research work from Vieytes et al., 1997 [6]. This test uses the inhibitory activity of OA and DTXs against the protein phosphate enzyme, which is responsible for their toxic effect, for the detection of OA-toxins group in molluscs. The original kit was recently modified into a new kit version, the colorimetric TOXILINE-DSP. This new kit is still based on the inhibitory capacity of the phosphatase enzyme on the OA and DTXs, but uses a colorimetric substrate [7] instead of the fluorimetric one used in the original method [6] to detect the reaction of inhibition.

The colorimetric TOXILINE-DSP.Co test has been Single-Laboratory Validated by ZEU-INMUNOTEC and the EURLMB (European Reference Laboratory for Marine

Biotoxins). Table 1 shows a summary of the results obtained by the manufacturer. Those obtained by the EURLMB will be available at their website soon (*http://www.aesan.msc.es/en/CRLMB/web/home.shtml)*.

A pre-validation study (small-scale interlaboratory comparison with four labs) was also carried out to obtain performance data related to the between laboratory reproducibility of the colorimetric TOXILINE-DSP test (Table 1) (See also reference documents, *TOXILINE-DSP.Co. Pre-validation Study report: ED-Pre-Validation-Report-TOXILINE DSPcol-001 Rev03*).

A method comparison with the current (Mouse Bioassay, MBA) [8] and future Liquid Chromatography coupled to mass spectrometry, (LC-MS/MS) [9] reference method was also carried out as part of the Single-Lab Validation (SLV) and pre-validation studies. Individual results are shown in the TOXILINE-DSP.Co. Single Validation Report (SLV Toxiline-DSP.Co, G-COM-OA.09. Rev. 2) and TOXILINE-DSP.Co. Pre-validation Study Report (ED-Pre-Validation-Report-TOXILINE DSPcol-001 Rev03). A summary of these results can be found in table 2.

Table 1. Summary of performance characteristics of the TOXILINE-DSP.Co test obtained during the single-laboratory validation study carried out by the manufacturer. * Data from pre-validation study**.**
RSDr Repeatability Relative Standard Deviation. RSDR: Reproducibility Relative Standard Deviation

Parameter	**Result**	
Limit of detection (LOD) n=8. Matrix: mussel	44 µg OA equivalents /kg (blank sample + 3SD)	
Limit of Quantification (LOQ) n= 8. Matrix: mussel	56 µg OA equivalents /kg (blank mussel + 10SD)	
Calibration curve	0.5 nM-2.8 nM	
Working range	from 63 to 352 µg OA equivalents/kg	
Precision: **repeatability** Matrix: mussel	**Level (µg OA equiv./kg)**	**RSDr** (n=8)
	124	3.9%
	276	1.4%
Within-laboratory reproducibility (intermediate precision) Matrix: mussel	**Level (µg OA equiv./kg)**	**RSDr** (n=3)
	88	7.0%
	122	7.8%
	282	2.4%
Accuracy: Recovery, % Spiked with OA Matrix: mussel	**Spiked Level (µg OA equiv./kg)**	**Recovery (RSDr for n=5)**
	80	101% (14.6%)
	160	90% (8.9%)
	240	78% (5.4%)
Accuracy: Recovery, % Spiked with OA Matrix: scallop	**Spiked Level (µg OA equiv./kg)**	**Recovery (RSDr for n=5)**
	80	114% (9.9%)
	160	98% (8.4%)
	240	106% (8.7%)
Accuracy: Recovery, % Spiked with DTX 1 Matrix: scallop	**Spiked Level (µg OA equiv./kg)**	**Recovery (RSDr for n=5)**
	80	102% (14.5%)
	160	79% (11.7%)
	240	88% (16.9%)
Accuracy: Recovery, % Spiked with DTX 2 Matrix: mussel	Spiked **Level (µg OA equiv./kg)**	**Recovery (RSDr for n=5)**
	80	93% (2.3%)
***Estimate of between-laboratory reproducibility** 4 laboratories 7 samples including naturally contaminated samples (mussle, cockle, cooked mussel and donax) Blank samples included	**Level (µg OA equiv./kg)**	**RSDR**
	70-250	<12%

Note. The SLV was carried out with the SOP Rev0.

Table 2. Results from TOXILINE-DSP.Co, MBA [8] and LC-MS/MS [9]. Of the 37 samples only 31 were tested by MBA. Positive results (+): ≥160 µk/kg. Negative result (-): <160 µk/kg.

	Coincident results	False Positive	False Negative
TOXILINE DSP.Co vs MBA 31 samples	94 %	0%	6%
TOXILINE DSP.Co vs LC-MS/MS 37 samples	84%	11%	5%
LC-MS/MS vs MBA 31 samples	84%	0%	16%

2. TEST METHOD

TOXILINE DSP.Co is an enzymatic test for quantitative determination of Okadaic Acid (OA) and other carboxylic toxins of the OA group, including DTX1, DTX2 and their ester forms, based on a colorimetric phosphatase inhibition assay. This method is applicable to shellfish species such as mussels, clams, cockles and scallops.

The toxicity of OA and DTXs is directly related to their inhibitory activity against a family of structurally related serine/threonine protein phosphatases (PP), in particular PP1 and PP2A. This strong inhibitory property is used to determine OA content in shellfish by means of a microtiter plate assay, using the enzyme PP and a chromogenic substrate for this enzyme. The enzyme hydrolyses the substrate and the product can be measured by an absorbance measurement at 405 nm using a microplate reader. As the ability of the PP to hydrolyse the substrate depends on the presence of OA and analogues in the samples, the toxin concentration can be calculated by using a standard curve.

TOXILINE-DSP.Co includes five OA standards (0.5, 0.8, 1.2, 1.8 and 2.8 nM), phosphatase enzyme and substrate reagents ready to use; and it has a working range from 63 to 352 µg OA equivalents/kg.

The test method is extensively described in the respective Standard Operational Procedure (S. O. P. TOXILINE DSP.Co G-COM-OA 07. Rev 3).

3. PURPOSE

The main purpose of the collaborative study is to determine repeatability and between-laboratory reproducibility of the TOXILINE-DSP colorimetric test (TOXILINE-DSP.Co) for the detection of the Okadaic acid-toxins group (OA-group) in molluscs.

A recovery experiment was also carried out in this study, to determine the accuracy of the method.

4. TEST MATERIALS

4.1. DESCRIPTION OF THE TEST MATERIALS

Eight different test materials, as blind duplicates (total of sixteen samples), were tested by each laboratory in this study. Five materials contained different OA-toxins levels (within the working range of the test), both naturally contaminated and spiked. Three of them were blank samples.

An extra blank test material was also used in a recovery study.

The test materials belonged to four different types of molluscs (*Mytilus sp, Pecten sp. Venerupis sp. and Cerastoderma sp.)* and seven different species. Details of the materials used are shown in Table 3.

Table 3. Details of matrices, species and origin of the testing materials used in this study.

Test Material	CODE	Matrix/ Specie	Origin	Preparation Date
1	**A**	Mussel (*Mytilus galloprovincialis)*	Galicia, NW of Spain	25/08/2010
2	**D**	Clam *(Venerupis pullastra)*	FAO 37 Mediterranean sea	25/08/2010
3	**E**	Mussel *(Mytilus galloprovincialis)*	Galicia, NW of Spain	06/09/2010
4	**F**	Scallop *(Pecten maximus)*	FAO 27 NE Atlantic	25/08/2010
5	**G**	Clam *(Venerupis decussatus)*	Galicia, NW of Spain	06/09/2010
6	**K**	Clam *(Venerupis romboides)*	Galicia, NW of Spain	05/10/2010
7	**L**	Cockle *(Cerastoderma edulis)*	Portugal & Galicia, NW of Spain	08/10/2010
8	**N**	Mussel *(Mytilus edulis)*	Ireland	05/10/2010
	BM	Scallop *(Pecten maximus)*	Scotland	09/06/2010

4.2. PREPARATION OF THE TEST MATERIALS

The test samples were prepared by ANFACO-CECOPESCA. ZEU-INMUNOTEC processed the blank material (BM) used in the recovery experiment.

Materials A, E, D, and K were purchased fresh and alive. They were thoroughly cleaned outside and inside with fresh water to remove sand and any other foreign materials. Tissues were removed from the shell, transferred to strainers and drained for five minutes before homogenization (blender + Ultraturrax). The homogenate (at least 450g) was then distributed into plastic containers of 5.0 g ± 0.1 g, frozen and stored at approx. –24ºC until analysis or day of shipment.

Materials G and F were purchased frozen from the retail market. They were thawed at room temperature, and then cleaned and prepared as described above.

Material L was provided cleaned, blended and frozen by the European Union Reference Laboratory for Marine Biotoxins. The sample contained OA+DTX2 and traces of DTX1. The sample was mixed with fresh cockle from the same specie *(Cerastoderma edulis)* and without toxin (from Portugal) prior being spiked with DTX1 (Wako Chemicals, Germany), in order to achieve a suitable toxin profile. First of all the sample was thawed out at room temperature, mixed with the blank material and spiked. Then, it was distributed into plastic containers of 5.0 g ± 0.1 g, frozen and stored at approx. –24ºC until day of shipment.

Material N was provided cleaned, blended and frozen by the National Reference Laboratory of Ireland. The sample had a high level of OA-group toxins, so it was mixed with mussel *(Mytilus edulis)* without toxin purchased in ~~North~~ Ireland, in order to achieve a suitable toxin concentration. The sample was thawed out at room temperature, mixed and then distributed into plastic containers of 5.0 g ± 0.1 g, frozen and stored at approx. –24ºC until day of shipment.

All the test materials were first analysed by TOXILINE DSP.Co and LC-MS/MS to determine the content of OA toxins. Results obtained by both methods for samples A, F and G showed concentration for OA toxins below their limit of detection (40 and 45 µg/kg, respectively). The BM material had been previously tested by LC-MS/MS at EURLMB and no peaks were detected for this group of toxins (LOD for this method 15 µg/kg) [9]. Therefore, materials A, F, G and BM were considered blank and homogeneity or stability tests were not carried out.

Analyses by LC-MS/MS were also used to identify the toxins content and to ensure that all toxins belonging to the OA group were present in the materials.

Materials D, E, K, L and N were quantify by ANFACO and EURLMB [10] using LC-MC/MS. Table 4 shows concentration in OA equivalents and toxins profile of the different materials used.

Table 4. Total concentration of OA-toxins group (µg/Kg) determined by TOXILINE DSP.Co and LC-MS/MS, and toxins profile. Total content determined by LC-MS/MS was carried out at EURLMB [10] and ANFACO, and the toxicity factors were applied. Samples presented in increasing order of concentration by TOXILINE-DSP.Co.

CODE	MATRIX	TOTAL OA equivalents (µg/kg)			OA TOXINS CONTENT
		TOXILINE-DSP.Co(3)	LC-MS/MS(4)	LC-MS/MS(5)	
BM	**Scallop** *Pecten maximus*	< LOD	< LOD	< LOD	-
A	**Mussel** *Mytilus galloprovincialis*	< LOD		< LOD	-
F	**Scallop** *Pecten maximus*	< LOD		< LOD	-
G	**Clam** *Venerupis decussatus*	< LOD		< LOD	-
E	**Mussel** *Mytilus galloprovincialis*	78.9 ± 5.24	53.1	*109.5*	*OA*
L	**Cockle**(1) *Cerastoderma edulis*	168 ± 11.3	**110.6** 50.7(OA), 42.7(DTX1), 28.6 (DTX2)	*< LOQ*	*OA, DTX1 & DTX2*
D	**Clam** *Venerupis pullastra*	240 ± 9.33	159.7	*278*	*OA*
K	**Clam** *Venerupis romboides*	250 ± 6.57	186.8	*223*	*OA*
N	**Mussel**(2) *Mytilus edulis*	276 ± 6.48	**166.6** 94.9 (OA) 119.5 (DTX2)	*183.4 106 (OA) 77.4 (DTX2)*	*OA & DTX2*

(1) Artificially contaminated with DTX1 and mixed with blank material. (2). Mixed with blank material

(3) Average of OA equivalents obtained from the homogeneity study (4). LC-MS/MS analysis carried out by EURLMB. (5) LC-MS/MS analysis carried out by ANFACO. Peaks for OA and DTX1 were identified in material L, but levels found were below the LOQ (40 µg/kg) for each of them.

4.3. HOMOGENEITY OF THE TEST MATERIALS

Homogeneity and stability of materials D, E, K, L and N were studied at ZEU-INMUNOTEC. According to the International Harmonized Protocol for the Proficiency Testing of Analytical Chemistry Laboratories [11], ten containers of 5 g were randomly selected. Then, each container was homogenized, extracted and two test portions (from the sample extract) were analysed to estimate the analytical variance. A total of 20 test portions per material were tested under repeatability conditions and in a random order, using TOXILINE DSP.Co test kit (G-COM-OA.07. Rev 2).

Cochran´s test for duplicate results was applied to all data obtained and no outliers were detected at the 95% level of confidence. To determine the suitable homogeneity of the materials, variance of the sums (Vs) of pairs of duplicates, the analytical variance (s^2_{an}) and the sampling variance (s_{sam}^2) were calculated. The allowable sampling variance (σ^2_{all}) was determined as $\sigma^2_{all} = (0.3\ \sigma_p)^2$, being σ_p the standard deviation for proficiency assessment [12]. Such a standard deviation was set as 13% from the

results obtained in the precision study carried out to estimate between-laboratory reproducibility during the single-laboratory validation (Table 1).

The critical value for the test, $c = F_1\sigma^2_{all} + F_2 s^2_{an}$, was assessed (factors F_1 and F_2 are tabulated values at the 95% level of confidence and m = 10 samples measured in duplicate). If $s_{sam}^2 > c$, there is evidence (significant at the 95% level of confidence) that the sampling standard deviation in the population of samples exceeds the allowable fraction of the target standard deviation, and the test for homogeneity has failed. If $s_{sam}^2 < c$ there is no such evidence, and the test for homogeneity has been passed.

Results obtained for sufficient homogeneity for Materials D, E, K, L and N are shown in Table 5.

Table 5. Homogeneity test for test materials for the determination of okadaic acid (µg OA total equivalents/kg).

TEST MATERIAL	Variance of sums, Vs	Analytical Variance, s_{an}^2	Allowable sampling variance, σ_{all} ^2	Sampling Variance, S_{sam}^2	Critical Value, c	Test for homogeneity result
D	166	90.7	36.8	116	310	S_{sam}^2 < c
E	84.7	8.09	19.8	11.1	29.1	S_{sam}^2 < c
K	139	19.6	32.5	126	257	S_{sam}^2 < c
L	356	46.9	85.7	55.6	152	S_{sam}^2 < c
N	124	24.2	28.4	154	314	S_{sam}^2 < c

4.4. STABILITY OF THE TEST MATERIALS

To ensure the stability of the materials during their distribution to participants and the study duration, aliquots of each material were taken randomly and split into two subsets, each of them containing 5 samples. One subset was used as control and stored at - 18 ± 1 ºC. The second one was stored under experimental conditions of 9.0 ºC ± 1 ºC for 5 days. Both subsets were randomized before testing and analysed simultaneously using TOXILINE-DSP.Co under repeatability conditions. The means, standard deviations and differences between both subsets were calculated and analysed by studying their variance (f Test>0.1 is pass) and performing a t Test (t Test > 0.05 is pass). No statistical differences between the two subsets could be identified. The absolute difference (D) was compared to C (The standard deviation for proficiency

assessment [σ_p] set at 0.13 [i.e., an RSD of 13 %] multiplied by the mean concentration of the control sample). All samples applied with the criterion (D<C) (Table 6).

Table 6. Results obtained for the stability assays conducted in materials D, E, K, L and N.

	Storage Conditions						
	- 18 ± 1 ºC	**9.0 ± 1 ºC**					
TEST MATERIAL	**Mean**	**Mean**	**Absolute Difference D**	**Variance f Test**	**t Test**	**Test Criterion C**	**D < C**
	Total OA euqivalents (µg/kg)						
D	265 ± 10.4	262 ± 14.5	3.02	0.54	0.71	34.5	pass
E	84.0 ± 4.34	85.1 ± 2.90	-1.19	0.45	0.62	10.9	pass
K	255 ± 8.03	257 ± 7.34	-1.57	0.87	0.75	33.2	pass
L	171 ± 6.81	169 ± 7.85	1.63	0.79	0.73	22.2	pass
N	343 ± 24.0	355 ± 32.2	-13.0	0.58	0.49	44.6	pass

5. ORGANIZATION OF THE VALIDATION STUDY

5.1. STUDY PLAN

A Validation Management Team (VMT) was appointed to supervise, advise and ensure the independence of the study.

A full description of the validation study, where details of the test method, experimental design, preparation of test materials, instructions for participants, key personnel, schedule, etc, is included in the Study Plan (G-COM-OA 08 Rev 2)**.** This document was agreed by the VMT under the lead of study coordinator; and provided to all participants prior distribution of the test materials.

5.2. PARTICIPANTS

A total of 16 participants took part in the validation study. The study plan however shows only 15 laboratories. A new lab was interested in the study, once the planning was finalised and materials delivered to participants. After consultation with the VMT, it was decided to accept this last collaborator, although deadline for registration was ended.

The participants are potential end-users of the TOXILINE DSP.Co test since most of them perform the official control for marine biotoxins. They represent 11 countries (9 of

them European); their affiliation and contact persons during the validation are summarised in table 7.

Table 7: Participant laboratories in the validation study of the TOXILINE-DSP.Co Test.
EURLMB: European Reference Laboratory for Marine Biotoxins. NRL: National Reference Laboratory. Participants are listed in alphabetical order.

LABORATORY	ADDRESS	RESPONSIBLE	Contact e-mail
ANFACO-CECOPESCA	Campus Universitario Vigo 36310 Vigo (Pontevedra) **SPAIN** Tel. +34 986469303 Fax. +34 986469269	Alberto Otero	aotero@anfaco.es
CEFAS	Centre for Environment Fisheries and Aquaculture Science Barrack Road, Weymouth, Dorset, DT4 8UB-**UNITED KINGDOM** Tel: +44 1305 206636 Fax: +44 1305 206601	Andrew Turner	andrew.turner@cefas.co.uk
EURLMB-AESAN	Estación Marítima S/N. Muelle de Trasatlánticos 36200 Vigo- **SPAIN** Tel: +34 986443340 Fax: +34 986229956	María Luisa Rodríguez Velasco	mrodriguezv@mspsi.es
INTECMAR	Peirao de Vilaxoán, s/n 36611 Vilagarcía de Arousa (Pontevedra)-**SPAIN** Tel. +34 986512320/22 Fax. +34 986512300	Jorge Correa	Jcorrea@intecmar.org
IRTA	Ctra. Poble Nou, km 5,5 Sant Carles de la Ràpita Tarragona- **SPAIN** Tel. +34 977 745 427 Fax. +34 977 744 138	Mònica Campàs	monica.campas@irta.cat
LGL	Bayrisches Landesamt für Gesundheit und Lebensmittelsicherheit (LGL) Chemische Untersuchung von Milchfetten, Fischen und Eiern D-91058 Erlangen- **GERMANY** Tel. +49 (0) 9131-764 347 Fax. +49 (0) 9131-764 601	Ulrich Schwank	Ulrich.Schwank@lgl.bayern.de
NRL- Germany	Bundesinstitut für Risikobewertung (BfR- Federal Institute for Risk Assessment) Thielallee 88-92 14195 Berlin- **GERMANY** Tel:+ 49 30 8412 3299 Fax: + 49 30 8412 3457	Katrin Kapp	katrin.kapp@bfr.bund.de
NRL -Greece	3A Limnou street 54627 Thessaloniki- **GREECE** Tel: +30 2310 552928 Fax: +30 2310 566581	Panagiota Katikou	biotoxin@otenet.gr

NRL -Ireland	Marine Environment & Food Safety Services, Marine Institute, Rinville. Oranmore, Co. Galway –**IRELAND** Tel: + 353 91 387238 Fax: + 353 91 387237	Dave Clarke	dave.clarke@marine.ie
NRL-Latvia	Institute of Food Safety, Animal Health and Environment „BIOR” Lejupes Street 3 Riga-**LATVIA** Tel: +371 67620513 Fax: +371 67620434	Guntis Cepurnieks	guntis.cepurnieks@bior.gov.lv
NRL- Poland	National Veterinary Research Institute Ail. Partyzantòw 57 24 - 100 Pulawy**- POLAND** Tel: +48 81 8893184 Fax: +48 81 8862595	Mirslaw Michalski	mmichal@piwet.pulawy.pl
NRL- Portugal	INRB/IPIMAR Av. Brasília, s/n., 1449-006 Lisboa - **PORTUGA**L Tel: +351-213027125 Fax: +351-213015948	Paolo Vale	pvale@ipimar.pt
NRL-Romania	The Institute for Diagnosis and Animal Health 63 Dr, Staicovici Street, sect.5, Bucharest- **ROMANIA** Tel: +40 374322029 Fax: +40 214113394	Vlad Serafim	serafim.vlad@idah.ro
PUBLIC HEALTH INSTITUTE OF CHILE	Marathon, Ñuñoa, Santiago de Chile -**CHILE** Tel: +56-2-5755492 Fax: +56-2-3507489	Lorena Andrea Delgado	ldelgado@ispch.cl
SENASA Mar de Plata City Regional Laboratory	Av. Dorrego y Víctimas del ’46, Banquina Puerto Mar de Plata, Buenos Aires- **REP. ARGENTINA** Tel: 54 223 480 2226 Fax: 54 223 480 2226	Alejandra Goya	agoya@senasa.gov.ar
ZEU-INMUNOTEC	C/Bari., 25 Dpdo. Poligono Plaza, 50197- Zaragoza-**SPAIN** Tel: +34 976 731 533 Fax: +34 976 524 078	Henry Smienk	hsmienk@zeulab.com

5.3. DISTRIBUTION OF THE TEST MATERIALS AND REAGENTS

The test materials were blind coded by EURLMB and distributed from ANFACO-CECOPESCA to the participants. The codes were securely kept by EURLMB until statistical analysis of results was carried out.

The materials were shipped in isothermal boxes with dried ice and were received within the following two days by most participants. Materials sent to Chile and Argentina were delivered over a week later from the dispatch date, as these countries have a long customs check up procedures. Samples were however reported to be kept frozen while stored at customs.

Each participant received sets 1 and 2 with 9 and 7 samples, to be tested on days 1 and 2, respectively. There was also a third set with 2 samples to carry out the recovery experiment. An extra sample was also supplied for the participants to run a familiarization assay.

The kit reagents together with the familiarization sample and spiking solution were shipped in isothermal boxes from ZEU-INMUNOTEC, arriving to participants within similar lead times described for the samples. Each participant received enough reagents to carry out all the requested assays.

All participants filled in an arrival form (Annex III of the Study Plan) with details of the materials and reagents received. All packages contained the right material for the study, although 2 labs informed that the box containing the samples did not arrive in good conditions and 6 labs reported samples to be cold, but defrosted.

EURLMB checked the samples codes received by each participant and confirmed that they were correct.

5.4. ASSAYS SCHEDULE

Participants were given specific instructions on what samples to run each day of the study and the layout of samples and duplicates in the microtiter plate (see working plan, Annex IV of the Study plan).

A Standard Operational Procedure (SOP Toxiline-DSP.Co G-COM-OA 07. Rev 3) with description of the reagents preparation, sample extraction, test protocol and recovery experiment was provided and strictly followed by the laboratories. No deviations were reported or identified.

5.5. SUBMISSION OF DATA AND REPORTING

All participants sent back an electronic copy of the Reporting Sheet (Annex V of the Study plan) for each day of analysis, with the raw data and final results for each test material. A copy of lab results was forwarded to EURLBM prior processing the data to guarantee that only data provided was used.

The reporting sheets were checked at reception for obvious errors in sample codes and calculations.

Participants also completed a questionnaire with details of the equipment used, preparation of reagents and samples and feedback of the assay.

6. RESULTS AND DISCUSSION

6.1. ANALYSIS OF VALID DATA AND OUTLIERS

The results obtained by each laboratory per test material and day of analysis are shown in table 8. Statistical data analysis was carried out following the approach described in the AOAC/IUPAC guidelines [11, 12, 13].

The data was first analysed for possible outliers applying the Cochran and Grubbs tests. Then, precision parameters, HORRAT values and % of recovery were calculated (tables 9 and 10).

The results were initially reviewed to remove invalid data. Results from assays with calibration curves with a $R^2 < 0.96$, outside the working range or showing deviations from the S.O.P. would have been considered invalid.

Two laboratories reported one of the assays with $R^2 < 0.96$, one of them (Lab A) repeated the analysis obtaining R^2 within the required criterion. Laboratory L however, could not repeat the assay on time and those results were considered invalid and removed for statistical analysis. For Lab A the reason for the assay failure was determined to be insufficient phosphatase reconstitution. In case of Lab L the causes are being investigated.

Materials A, F and G were not statistically analysed as they were blank samples. However, laboratory J reported values within the working range of the test for materials A and G. These values are considered incorrect according to the AOAC guidelines [12], as they are positive values found for a blank material. All the other labs in the study identified the blank materials below the working range of the test. The manufacturer is investigating with the laboratory possible causes of this disagreement.

The valid data from the contaminated test materials (D, E, K, L and N) was then analysed for identification of outliers following the Cochran and Grubbs tests as described below.

Results from laboratory L could not be included in the statistical analysis as only one value per material was available.

Cochran test, to remove laboratories (or indirectly for removal of extreme individual values from a set of laboratory values) showing significantly greater variability among replicate (within-laboratory) analyses than the other laboratories for a given material. A 1-tail test at a probability value of 2.5% was applied.

To calculate the Cochran test statistic, the within-laboratory variance for each laboratory and each material was computed and the largest of these was divided by the sum of all those variances, and multiply by 100. The resulting quotient was the

Cochran statistic which would indicate the presence of a removable outlier if this quotient exceeded the critical value list in the Cochran table [11, 12] for P = 2.5% (1-tail) and L (number of laboratories). For 15 laboratories and duplicate analysis of each material the critical value assigned was 51.5.

This test showed lab G for material K and lab P for material E as outliers. The Cochran test was then applied again to the results, once these outliers were removed. Lab J for Material E was also excluded in a second round.

Grubbs test, to remove laboratories with extreme averages. This test was applied to the remained values from the Cochran test. A single value test (2-tail, P = 2.5%) was first applied, followed by pair value test (2 values at the highest end, 2 values at the lowest end, and 2 values, one at each end, at an overall P = 2.5%).

To calculate the single Grubbs test statistic, the average of the values obtained for each material by each laboratory was computed; then the standard deviation (SD) of those L averages was calculated and designated as the original s. The SD of the set of averages with the highest average removed was calculated, and designated as s_H; the SD of the set averages with the lowest average removed was calculated, and designated as s_L. The percentage decrease in SD was then calculated as follows: 100 x [1- (s_L/s)] and 100 x [1- (s_H/s)].

The higher of these 2 percentage decreases was the single Grubbs statistic, which would signal the presence of an outlier to be omitted if it exceeded the critical value listed in the single Grubbs tables [11, 12] at the P = 2.,5% level, for L laboratories.

As no outlier was identified by applying single Grubbs test, the Grubbs pair statistic was calculated in an analogous way, but calculating s_{2L}, s_{2H} and s_{HL}, following removal of the 2 lowest, the 2 highest, and the highest and the lowest averages, respectively, from the original set of averages. In this case, by taking the smallest of those three SD values, the corresponding percentage decrease in SD from the original s was calculated. A Grubbs outlier pair would be presented if the selected for the percentage decrease from the original s exceeded the critical value listed in the Grubbs pair value table [11, 12], at the P = 2.5% level, for L laboratories.

No outliers were identified by the Grubbs pair value test.

Individual results on days 1 and 2 for material L from labs J and P showed large differences comparing to those obtained by the rest of the labs. However, they were not identified as outliers, as the presence of a second lab with poor reproducibility has a great impact on the sum of the variances and prevents the elimination of the first lab.

Table 8 shows in grey the materials identified as outliers and therefore excluded for further calculations.

Table 8. Individual results (µg OA total equivalents/kg) reported from labs A to P for Material A, D, E, F, G, K, L and N on days 1 and 2. The outlier values identified by the Cochran test and therefore not included in statistical analysis are shown in grey. Invalid or incorrect results are those crossed out.

	MATERIAL A		MATERIAL D		MATERIAL E		MATERIAL F		MATERIAL G		MATERIAL K		MATERIAL L		MATERIAL N	
	DAY 1	DAY 2	DAY 1	DAY 2	DAY 1	DAY 2	DAY 1	DAY 2	DAY 1	DAY 2	DAY 1	DAY 2	DAY 1	DAY 2	DAY 1	DAY 2
	µg OA total equivalents/kg															
LAB A	<63	<63	186	239	97	102	<63	<63	<63	<63	248	281	167	174	210	247
LAB B	<63	<63	251	266	100	101	<63	<63	<63	<63	302	299	177	190	273	277
LAB C	<63	<63	244	233	96	87	<63	<63	<63	<63	279	246	174	160	256	251
LAB D	<63	<63	264	253	125	100	<63	<63	<63	<63	282	277	189	223	269	295
LAB E	<63	<63	210	233	101	120	<63	<63	<63	<63	239	244	156	181	226	219
LAB F	<63	<63	252	250	113	116	<63	<63	<63	<63	287	286	166	165	271	275
LAB G	<63	<63	246	252	89	100	<63	<63	<63	<63	356	269	192	192	274	236
LAB H	<63	<63	253	250	90	99	<63	<63	<63	<63	291	301	175	179	271	270
LAB I	<63	<63	252	254	95	87	<63	<63	<63	<63	284	283	169	161	265	253
LAB J	~~70~~	~~98~~	238	239	163	102	<63	<63	~~78~~	~~67~~	248	268	239	184	246	235
LAB K	<63	<63	253	264	81	81	<63	<63	<63	<63	295	300	152	160	247	266
LAB L	~~-~~	~~<63~~	~~-~~	~~242~~	~~-~~	~~145~~	~~-~~	~~<63~~	~~-~~	~~-~~	~~-~~	~~266~~	~~-~~	~~202~~	~~-~~	~~182~~
LAB M	<63	<63	257	255	101	104	<63	<63	<63	<63	292	274	177	176	271	272
LAB N	<63	<63	261	251	98	101	<63	<63	<63	<63	285	285	161	181	257	250
LAB O	<63	<63	221	223	91	94	<63	<63	<63	<63	270	249	179	184	259	244
LAB P	<63	<63	192	241	69	153	<63	<63	<63	<63	226	278	97	173	206	259

6.2. PRECISION

To estimate the precision of the method the within-laboratory repeatability and between-laboratory reproducibility were determined by calculated s_r (repeatability standards deviation), s_R (reproducibility standard deviation), relative standard deviations (RSDr and RSDR), repeatability and reproducibility limits and HORRAT values. All these values are shown in table 9.

Following the AOAC guidelines [12] those parameters were computed as indicated below:

Repeatability standard deviation:

$$s_r = (\text{sum}d_i^2/2L)^{1/2},$$

where d_i is the difference between the individual values for the pair in laboratory i and L is the number of laboratories or number of pairs.

Reproducibility standard deviation:

$$s_R = (1/2(s_d^2+s_r^2))^{1/2}$$

where $s_d^2 = \text{sum}(T_i-\bar{T})^2/(2(L-1))$, being T_i the sum of the individual values for the pair in laboratory i, $\bar{T}$ the mean of the T_i across all laboratories of pairs, L the number of laboratories or pairs, and s_r^2 is the square of s_r.

In order to facilitate comparison of variabilities for different test materials included in the study, the relative standard deviation (RSD) under repeatability (RSD_r) and reproducibility (RSD_R) conditions were calculated as follows:

$$RSD_r\ (\%) = 100s_r / \text{mean};\ \text{and}\ RSD_R\ (\%) = 100s_R / \text{mean}$$

Repeatability limit (r): is the interval representing the critical difference between two test results for the same material, obtained by the same operator using the same equipment on the same day in the same laboratory.

r: Sr x 2.8

Reproducibility limit (R):, is the interval representing the critical difference between two test results for the same material, obtained by different operators using different equipment in different laboratories.

R: S_R x 2.8

HORRAT:

$$HORRAT = RSD_R (\%) / PRSD_R (\%);$$

where $PRSD_R$ (%) = $2C^{-0.1505}$ and C = the estimated mean concentration assigned value (Kg okadaic acid equivalents / Kg material).

The overall mean values assigned for OA-toxins group for the test materials were 98.8, 175.4, 242.8, 255.0 and 275.0 µg total equivalents OA/kg for Materials E, L, D, N and K, respectively (Table 9).

Values obtained for repeatability standard deviation (Sr) ranged from 7.3 for Material E to 19.6 µg /kg for Material L, with repeatability relative standard deviations (RSDr) from 5.4% for Material K to 11.2% for Material L (Table 9).

The reproducibility standard deviation (S_R) calculated for the 5 test materials ranged from 10.7 to 23.2 µg/kg; with reproducibility relative standard deviation (RSDR) values from 7.6 % to 13.2 % for Materials K ad L, respectively (Table 9).

The HORRAT values obtained were 0.4 for materials D, K and N, 0.5 for material E and 0.6 for material L (Table 9) indicating a very good performance of the method. These values are just at the lower limit of the range considered as normally expected for a good reproducibility of a method (0.5 < HORRAT≤ 1.5), according to the AOAC guidelines [12].

All the individual values obtained per material, day and laboratory were also plotted. One graph per material is shown in figure 1. The solid lines represent the assigned mean value obtained for each material in this study (table 9) and the dotted lines show the mean value ± the theoretical reproducibility standard deviation ($PRSD_R$).

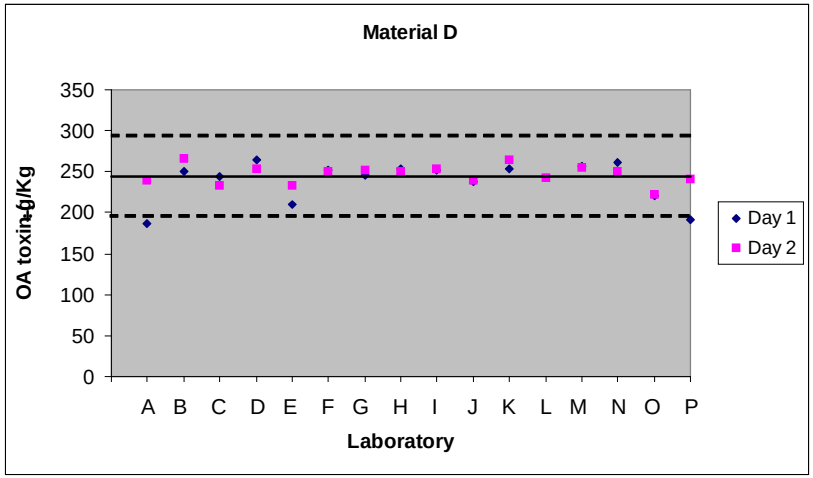
Table 9. Details of the test materials, number of results submitted and results after outliers, together with performance values of precision (repeatability and reproducibility) obtained for the colorimetric TOXILINE-DSP.Co.

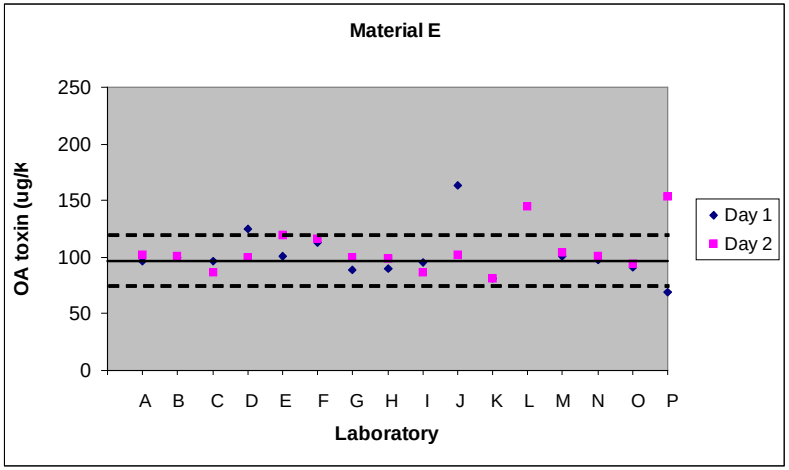
S_r: Repeatability standard deviation. S_R: Reproducibility standard deviation. RSD_r: Repeatability relative standard deviation. RSD_R: Reproducibility relative standard deviation. r: Repeatability Limit, R: Reproducibility Limit

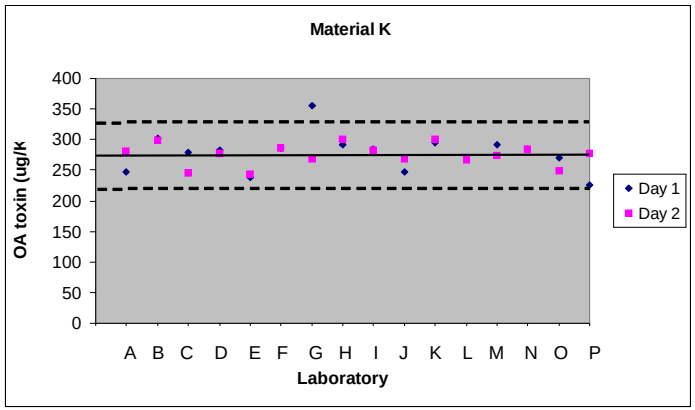
*See table 8 for values not included in statistical and precision analysis. a: number of laboratories remaining after removal of the number of outliers indicated by (b)

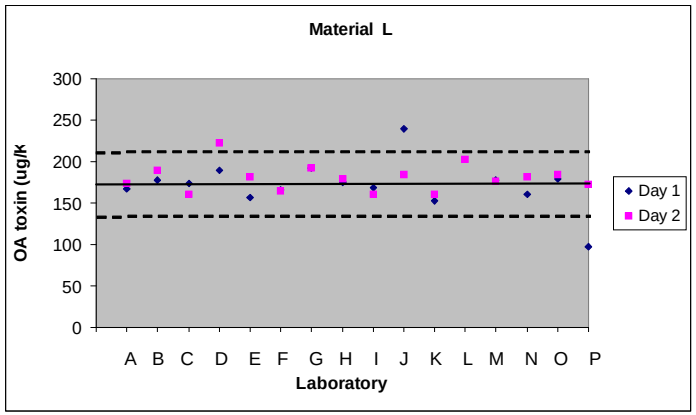
Test Material	Matrix	Runs/lab	No. labs submitting results	No. of labs after invalid/ Incorrect results*	No. of labs after outliers[a(b)]	Overall Mean (µg total equiv.OA/kg)	Repeatability			Reproducibility			
							µg total equiv.OA/kg			µg total equiv.OA/kg			
							S_r	r	RSD_r (%)	S_R	R	RSD_R (%)	HORRAT
A	**Mussel** *Mytilus galloprovincialis*	2	16	14	-	<63	-	-	-	-	-	-	-
D	**Clam** *Venerupis pullastra*	2	16	15	15 (0)	242.8	14.7	41.2	6.1	19.4	54.4	8.0	0.4
E	**Mussel** *Mytilus galloprovincialis*	2	16	15	13 (2)	98.8	7.32	20.5	7.4	10.7	30.0	10.7	0.5
F	**Scallop** *Pecten maximus*	2	16	15	-	<63	-	-	-	-	-	-	-
G	**Clam** *Venerupis decussatus*	2	16	14	-	<63	-	-	-	-	-	-	-
K	**Clam** *Venerupis romboides:*	2	16	15	14 (1)	275.0	14.9	41.8	5.4	21.0	58.7	7.6	0.4
L	**Cockle** *Cerastoderma edulis*	2	16	15	15 (0)	175.4	19.6	55.0	11.2	23.2	64.9	13.2	0.6
N	**Mussel** *Mytilus edulis*	2	16	15	15 (0)	255.0	15.6	43.7	6.1	20.7	58.1	8.1	0.4

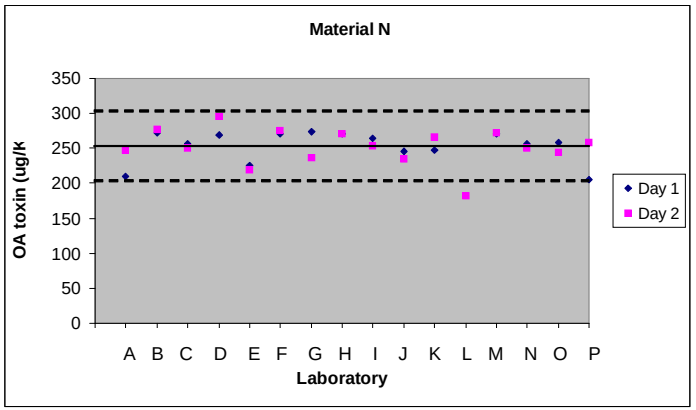
Figure 1. Individual results for each test material obtained per lab and per day of analysis (including outliers). The solid line shows the assigned mean value calculated in this study for each material. The dotted lines indicate the theoretical reproducibility standard deviation determined for each material in this study ($PRSD_R$).











6.3. RECOVERY

Although the main objective of the validation study was to determine the repeatability and between-laboratory reproducibility of the colorimetric TOXILINE-DSP.Co test for the detection of OA-group toxins in molluscs; a recovery assay was also carried out.

Due to the limited experience on the homogeneity and stability of samples spiked with OA toxins each participant prepared a spiked sample on the day of the assay. A blank material (BM) and an okadaic acid solution of known concentration (2 µM, to give a final concentration of 161 µg/kg) were provided to each participant for this experiment. BM and spiked sample (BM + OA) were analysed on day 2 of the collaborative study.

The BM sample was analysed by LC-MS and Toxiline-DSP.Co prior being included in the study. Results from LC-MS [9] at the EURLMB showed no peaks for this sample and therefore a concentration below the LOD (<15 µg OA equiv./Kg). TOXILINE-DSP.Co also produced results under its LOD (<44 µg OA equiv./Kg). So, the BM sample was then considered as blank material no containing OA toxins for calculations of the recoveries in this experiment.

For percent recovery calculations, the marginal percent recovery was calculated as follows:

$$\% \text{ Recovery} = 100\ (C_f - C_u)/C_A),$$

where C_f is the amount found for the fortified concentration, C_u is the amount present originally for the unfortified concentration, and C_A is the amount added.

Recovery values found were 71.6 % to 122.3% with a mean and relative standard deviation of 98.0 % and 14.5%, respectively (table 10). These recoveries are in agreement with the 70 to 125% range that is expected for this concentrations according to the AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals [14].

Table 10. Results from recovery experiment carried out during Day 2 of the collaborative study. BM: blank material, no okadaic acid toxins were detected and therefore it is considered a concentration of 0 for calculation purposes. BM + OA: concentration of the samples spiked with 161 µg/ Kg of OA solution.

LABORATORY CODE	µg OA total eq./kg			RECOVERY(%)
	BM*	Spiked Conc.	BM+OA*	
LAB A	-	161	172	107.1
LAB B	-	161	162	100.7
LAB C	-	161	155	96.3
LAB D	-	161	115	71.6
LAB E	-	161	124	77.3
LAB F	-	161	138	85.5
LAB G	-	161	162	100.7
LAB H	-	161	131	81.1
LAB I	-	161	152	94.4
LAB J	-	161	197	122.3
LAB K	-	161	152	94.4
LAB L	-	161	196	121.6
LAB M	-	161	153	95.0
LAB N	-	161	174	108.3
LAB O	-	161	155	96.3
LAB P	-	161	185	114.7
Mean Recovery %				**98.0**
SD				**14.2**
RSD %				**14.5**

*Raw results reported by participants were multiplied by a correction factor of 1.02 (25.5/25), as the final volume of the spiked samples was 25.5 ml instead of 25 ml used for the rest of the samples. The Reporting sheet provided calculated the results considering 25 ml and this has to be amended. This was noticed by one of the participants when assays had already carried out the assays.

6.4. COMENTS FROM PARTICIPANTS

Most participants reported in the questionnaire that the S.O.P. provided all the information they needed to perform the assay and that they did not have difficulties understanding any part of it.

Some comments were received by the participants via the reporting sheet or questionnaires.

Laboratory A informed about the phophatase solution not being fully hydrated when following the S.O.P. instructions. They use a non-orbital shaker for the preparation.

Laboratory F also reported that phosphatase had to be left for 15 extra min for full dissolution.

It seems that the use of a non-orbital shaker does not always guarantee full dissolution of this reagent. Manually mixing, longer preparation and final visual check of the solution should be included in the S.O.P.

Laboratory A and L had R^2 < 0.96 on day 1. Lab A requested more kit reagents to repeat the assay and a good calibration curve was obtained in the second attempt. Laboratory L could not repeat the assay again due to not time available to deliver kits and repeat assays.

Laboratory K reported some difficulties to use the adhesive strips to cover the plate, as they seem to be narrower than the strip plate. The manufacturer has taken noticed of this matter.

Laboratory P mentioned that providing information on the concentration of the familiarization sample and spiking solution would have been appreciated.

Laboratory O noticed that an extra 0.5 ml of volume, comparing with the other samples, was used in the recovery experiment. This was not taken in consideration when designing the reporting sheet and so calculation were not accurate. The recovery values obtained by the participants were amended considering the final volume used with the sample. This lab also requested to include in the S.O.P. the speed to be used for homogenization.

7. CONCLUSIONS

- The current legislation (Commission Regulation (EC) No 2074/2005) and Regulation No. 15/2011, that will be applied from July 2011, allow alternative and/or complementary assays to the reference one. These must be intralaboratory validated and successfully tested under a recognised proficiency test scheme.

- The precision and recovery values determine in this study for TOXILINE-DSP.Co can be considered acceptable for this type of methodology and the concentration range required.

- The colorimetric phosphate inhibition assay for determination of OA-group toxins in molluscs, TOXILINE-DSP.Co, could be proposed as alternative and/or complementary assay to the reference method for determination of the OA-group toxins.

8. REFERENCE DOCUMENTS

- **Standard Operation Procedure:** SOP-Toxiline-DSP-Co-G-COM-OA 07. Rev 3
- **TOXILINE-DSP. Co. Single Validation Report:** SLV Toxiline-DSP.Co, G-COM-OA.09. Rev. 2
- **TOXILINE-DSP. Co. Pre-validation Study report:** ED-Pre-Validation-Report-TOXILINE DSPcol-001 Rev03
- **Study Plan Toxiline.DSP.Co**, G-COM-OA 08 Rev 2

9. REFERENCES

1- Takai, A., Bialojan, C., Troschka, M. and Rüegg, J.C. ,1987. Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin. FEBS Lett. 217, 81-84.

2- Xing Y., Xu Y., Chen Y., Jeffrey P. D, Chao Y., Lin[1] Z., Li Z., Strack S.,. Stock J. B. and Shi Y. 2006. Structure of Protein Phosphatase 2A core enzyme bound to tumor-inducing toxins. *Cell*. 127, 2, 341 - 353.

3- REGULATION (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. *Official Journal of the European Communities*, L139, 55-255.

4- COMMISSION REGULATION (EC) No 15/2011 of 10 January 2011 amending Regulation (EC) No 274/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs. *Official Journal of the European Communities L6: 3-6.*

5- COMMISSION REGULATION (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. *Official Journal of the European Communities*, L338: 27-59.

6- Vieytes M.R., Fontal O.I., Leira J.M., Vieytes J.M., Botana L.M. 1997. A Fluorescent Microplate Assay for Diarrheic Shellfish Toxins. *Analytical Biochemistry*, 248, 258-264.

7- Takai A and Mieskes. Inhibitory effect of okadaic acid on the p-nitrophenyl phosphate phosphatase activity of protein phosphatases., *Biochem. J*. 275, pp 233-239, 1991.

8- Standard Operating Procedure for detection of Okadaic acid, Dinophysistoxins and Pectenotoxins by Mouse Bioassay. Harmonised EU MBA SOP Version 4.0 (April 2007). Available at: http://www.aesan.msps.es/CRLMB/docs/docs/metodos_analiticos_de_desarrollo/EU-Harmonised-SOP-MBA-Lipophilic-Version5-June2009.pdf

9. Multitoxin Reference Method for LC-MS analysis of okadaic acid, azaspiracid, yessotoxin, pectenotoxin and spirolide groups biotoxins. Standard Operating Procedure. SOP LC-MS June06 Rev01

10 EU-Harmonised Standard Operating Procedure for determination of Lipophilic Marine Biotoxins in molluscs by LC-MS/MS. Available at: http://www.aesan.msps.es/CRLMB/docs/docs/metodos_analiticos_de_desarrollo/EU-Harmonised-SOP-LIPO-LCMSM_Version2.pdf

11- Thompson M., Ellison S.L.R. and Wood R. 2006. The International Harmonized Protocol for Proficiency Testing of Analytical Chemistry Laboratories (IUPAC Technical Report). *Pure Applied Chemistry*, 78, 145-196.

12- Guidelines for collaborative study procedures to validate characteristics of a method of analysis (appendix D). 2005. AOAC International.

13- Horwitz W. 1995. Protocol for the design, conduct and interpretation of method-performance studies. *Pure Applied Chemistry*, 67, 331-333.

14- Horwitz W., AOAC 2002-12-19. Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals.

OkaTest

SINGLE LAB VALIDATION

REPORT

File Identifier: SLV -OkaTest, G-COM-OA.09. Rev. 3

Date of circulation: 27.06.13

Drafted by: Name Henry Smienk

Date: 15.12.10

Reviewed by: Name: Elena Dominguez

Date 26.06.13

Date of change/ Date of draft:	Version-number:	Changed page(s):	Summary of main change(s):	Changed by:
15.09.10	0	4	Correct the number of batches of phosphatase showed in 3.1 working range from 5 to 3	H. Smienk
15.09.10	0	13	Intermediate precision instead of reproducibilily	H. Smienk
15.12.10	1	Several pages	Correction of minor types (ej. OA by AO), Details on toxicity factors, table 22	H. Smienk
25.06.13	2	Several pages	Change name of kit, now OkaTest Update selectivity and applicability results. Uncerataintly Replace table 22, ver 2 for table 23 with new data	E. Domingez

OkaTest - SINGLE LAB VALIDATION REPORT

1- BACKGROUND

OkaTest is a test for detection of Okadaic Acid (OA) and other carboxylic toxins of the OA group including DTX1, DTX2 and DTX3 by a colorimetric phosphatase inhibition assay. It is applicable to shellfish species such as mussels, clams, oysters and scallops. It is a rapid and simple method suitable for quantitative determination of the OA- toxins group from 63 to 352 ug of OA equivalents / Kg including the maximum limit, established as 160 ug of OA equivalents /Kg in the Commission Regulation of 29 April 2004 (Regulation (EC) 853/2004).

The OKATEST test was developed by ZEU- INMUNOTEC based on the research work carried out by Vieytes et al. The method uses the inhibitory activity of OA and DTXs against the enzyme phosphate, which is responsible for their toxic effect, for the detection of OA-toxins group in molluscs. OkaTest uses a colorimetric detection system (Takai and Mieskes, 1991), while the original method (Vieytes et al., 1997) was based on a fluorimetric detection.

2- PRINCIPLE

OkaTest is a test based on the inhibition of phophatase activity by OA-toxins group, responsible for diarrheic shellfish poisoning (DSP). Under normal circumstances, a phosphatase enzyme is able to hydrolyse a specific substrate producing a reagent that can be detected by absorbance measurement (405 nm). Samples containing OA toxins will inhibit the enzyme activity proportionally to the amount of toxin contained in the sample. The concentration of toxin in the sample is calculated using a standard curve.

3- VALIDATION

To evaluate the performance of the **OkaTest kit**, the limit of detection, limit of quantification, precision, accuracy and other parameters were calculated according to Horwitz W., 1995 The homogeneity, stability and different variables affecting robustness were also evaluated. Finally, a method comparison was carried out.

3.1 Working range

The working range of the method should be more amply than the amount of standards present in the kit in order to prevent insufficient kit performance due to shifts in the laboratory conditions. Here, the working range is understood as the range of OA concentrations that do correctly adapt to the fitting procedure. The working range of the assay depends on the quantity and quality of the phosphatase present. Therefore, assays were performed with at least 3 different phosphatase batches and the "goodness of fit" was evaluated according to the kits´ specifications (R^2 > 0.96) with standard concentrations rising from 0.25 to 3.5 nM OA.

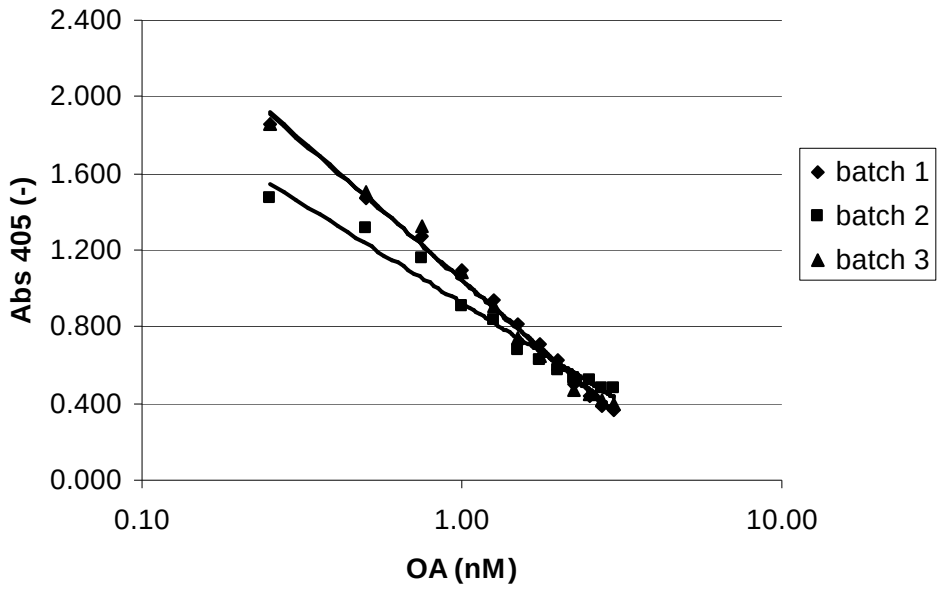


Fig 1. Working range of the assay for 3 different phosphatase batches. R^2: 0.99, 0.98 and 0.99 for batch 1, batch 2 and batch 3, respectively. Working range: 0.25, 0.5, 0.75.. etc.....3.0 nM OA.

Figure 1 shows the results of three assays covering the range from 0.25 to 3.0 nM OA as this was the range that always fitted correctly (R^2>0.96). This covers sufficiently the actual range of the standards in the kit (0.5 to 2.8 nM OA).

3.2 Linearity

The linearity of an assay is tested to find out whether the response of this assay is a function of the concentration of the analyte. The OkaTest assay uses a logarithmic fitting procedure.

As such the linearity of the assays' response was tested by 'backcalculation' of the standard concentration. For 'backcalculation' the equation of the standard curve is used to calculate the concentration of these standards from their absorbances (table 1). Another standard batch was introduced and the concentration calculated from the standard curve obtained with batch 1.

Table 1. Linearity of the assay. OA (nM) was calculated by using the standard curve of batch 1.

standards OA (nM)	batch 1 OA (nM)	batch 2 OA (nM)
0.5	0.6	0.5
0.8	0.7	0.7
1.2	1.1	1.2
1.8	1.9	1.9
2.8	2.9	2.8

To check the linearity of the response, the theoretical concentration was compared to the calculated concentration for both batches (see figure 2 for the results shown in table 1) and a linear fit was performed. The Pearson correlation coefficient (R^2) for batch 1 was 0.99 and 1.00 for batch 2.

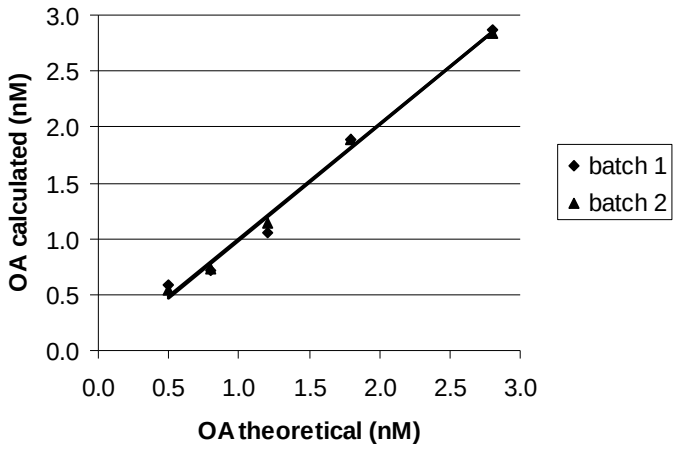


Fig 2. Comparison of the theoretical and calculated standard concentration. Concentration were `backcalculated´ by using the standard curve obtained with batch 1. R^2: 0.99 and 1.00 for batches 1 and 2, respectively.

3.3- Homogeneity and stability of the kit components

The homogeneity and/or stability of the components, critical for the kits´ performance, were tested. In case of the phosphatase, both the within batch and the between batch homogeneity and stability were tested, as each phosphatase vial is individually dispensed, liophilized and solubilized. The standards are presented ready-to-use and so only the between batch homogeneity and stability were tested.

3.3.1 Within batch homogeneity

To verify the homogeneity of the phosphatase, 5 vials were randomly picked from the same phosphatase batch. At least 5 different phosphatase batches were sampled this way. The assay was performed including internal control samples. These control samples were prepared by doping the diluent with okadaic acid at different concentrations, normally 95, 158 and 315 µg/kg OA. Table 2 shows the absorbances of the standard curves obtained with five different phosphatase vials.

Table 2. Data from 5 calibration curves collected on 5 different days. Mean, standard deviation (SD) and relative standard deviation (RSDr) were calculated. Internal control samples were used to verify correct quantification.

standards	**Vials (absorbance 405 nm)**							
OA (nM)	**1**	**2**	**3**	**4**	**5**			
0.5	1.977	1.645	1.650	1.567	1.618			
0.8	1.541	1.345	1.387	1.304	1.341			
1.2	1.306	1.049	1.069	0.959	1.019			
1.8	0.841	0.698	0.705	0.710	0.701			
2.8	0.552	0.496	0.492	0.509	0.524			
Control samples	**OA (µg/kg)**					**mean**	**SD**	**RSDr**
1	90	96	99	90	94	**94**	4.1	**4.4%**
2	154	165	161	159	168	**161**	5.4	**3.3%**
3	326	319	321	318	314	**319**	4.3	**1.3%**

All vials tested performed according to the tests specifications (R^2>0.96). The standard curves were used to calculate the OA concentration of the control samples. Finally, the mean and relative standard deviation were calculated. The relative standard deviation of the control samples was always below 5%.

3.3.2- Between batch homogeneity and stability

To test the between batch homogeneity of the phosphatase and at the same time the stability, a vial was randomly picked from five different batches at different shelf life stages. All vials were tested on the same day. Internal control samples (see also 3.1.2.1) were introduced to check for quantification (Table 3).

Table 3. Five different batches of phosphatase at different stages of shelf life. Mean, standard deviation (SD) and relative standard deviation (RSDr) were calculated. Internal control samples were used to verify correct quantification.

OA (nM)	Abs 405 nm							
	batch 1 2 months	batch 2 4 months	batch 3 8 months	batch 4 10 months	batch 5 12 months			
0.5	1.596	1.445	1.328	1.222	1.834			
0.8	1.362	1.212	1.126	0.967	1.601			
1.2	1.101	0.957	0.851	0.736	1.296			
1.8	0.652	0.626	0.506	0.589	0.821			
2.8	0.461	0.443	0.399	0.479	0.505			
Internal controls	**OA (µg/kg)**					**mean**	**SD**	**RSDr**
1	95	100	88.0	93.6	90	93	5	4.8%
2	160	169	162	156	144	158	9	6.0%
3	310	304	323	300	341	316	17	5.2%

The phosphatase showed good stability along the complete shelf life (9 months), as all assays performed were according to the kits specifications (R^2>0.96). The mean value of the samples was in accordance with the theoretical amount of okadaic present (control 1: 95 µg/kg, control 2: 158 µg/kg and control 3: 315 µg/kg). The relative standard deviation of the control samples was at the utmost 6%.

The between batch variation of the standards was tested accordingly. Five batches were chosen covering 90% of the shelf life of the component (6 months). These five standards were tested in one assay to be able to single out the variation due to the standards' stability and homogeneity (Table 4).

Table 4. Five different batches of standards at different stages of shelf life. The absorbances (405 nm) obtained for each of the standards are shown. Mean, standard deviation (SD) and relative standard deviation (RSDr) of these absorbances were calculated.

	Absorbance 405 nm							
Standards	batch 1	batch 2	batch 3	batch 4	batch 5			
OA (nM)	5 months	4 months	3 months	2 months	1 week	mean	SD	RSDr
0.0	2.042	2.100	2.064	2.073	2.120	2.079	0.031	1.5%
0.5	1.622	1.614	1.649	1.625	1.678	1.637	0.026	1.6%
0.8	1.462	1.390	1.386	1.375	1.372	1.397	0.037	2.7%
1.2	1.124	1.116	1.101	1.092	1.134	1.113	0.017	1.5%
1.8	0.772	0.792	0.769	0.822	0.809	0.793	0.023	2.9%
2.8	0.619	0.646	0.606	0.637	0.613	0.624	0.017	2.7%

No internal controls were added, as the variations of the standards were supposed to be small to have any effect on the quantification. A cero nM sample was added to be able to calculate the variation due to other variables than okadaic acid. The RSDr´s calculated from the absorbances were all within the same range. 1.5% of the variation seemed to be due to other variables than the okadaic acid present in the standards. All other RSDr´s were within the same range (<3%).

3.4- Ruggedness

The influence of different experimental conditions critical for the kits' performance such as assay temperature, incubation times or reaction component volumes were evaluated.

3.4.1- Assay temperature

The hydrolysis of the substrate by the phosphatase is temperature dependent and shows the typical behaviour of an enzymatic reaction with higher reaction rates close to the optimum temperature (37°C). However, a lower assay temperature was chosen to guarantee enzyme stability during the assay and to get stable reaction rates. The assay was tested at temperatures varying from 20 to 40 °C. 30 °C was chosen as the optimum temperature. At this temperature a 2 °C variation can be expected in any incubator. So, to show the influence of this temperature variation, 3 samples were quantified performing a complete assay (standard curve and samples) at each of these temperatures (Table 5).

Table 5. Influence of the assay temperature on the results of the test. The mean, standard deviation (SD) and relative standard deviation (RSDr) were calculated.

Sample	28 ºC	30 ºC	32 ºC	mean	SD	RSDr
1	104	100	97	100	3.4	3.4%
2	176	173	176	175	1.7	1.0%
3	302	303	298	301	2.6	0.9%

Mean and relative standard deviation were calculated. For all three samples RSDr were below the 15%, variation that can be expected at this concentration (Horwitz, AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals).

3.4.2- Assay incubation times

The assay consists of two different incubation steps that could affect the outcome of the test. During the first incubation the sample and the phosphatase are mixed, and the inhibition reaction should reach its endpoint. Following, the substrate is added and the plates are incubated for the second time. The main risk of this incubation step is phosphatase activity loss.

To determine the influence of time on the first incubation of the assay (normally 20 minutes), this step was varied between 18 and 24 min, while maintaining the rest of the assays' conditions according the kits' instructions. Three control samples were quantified and the variation in the relative standard deviation was evaluated. For each of the incubation conditions an independent assay was performed (Table 6).

Table 6. Influence of time on the first incubation of the assay. The mean, standard deviation (SD) and relative standard deviation (RSDr) were calculated.

Sample	- 2 min	0	+ 2 min	+ 4 min	mean	SD	RSDr
1	85	87	87	90	88	2.1	2.4%
2	152	155	161	164	158	5.7	3.6%
3	311	291	317	320	310	12.9	4.2%

In all cases the assay complied with the criterion (R^2>0.96). The relative standard deviations were comparable to those obtained when performing the test under standard conditions (highest 4.2%).

The second incubation was evaluated similarly. A 10% error from normal incubation time (30 minutes) was applied, adding some extra time (up to 20% or 6 minutes). The assay was performed as described; although no stopping solution was added to permit reading the same assay. The RSDr was 2.9% at highest, a bit lower than the ones obtained for the first incubation time (Table 7).

Table 7. Influence of the incubation time (2nd incubation with the substrate) on the assay. The mean, standard deviation (SD) and relative standard deviation (RSDr) were calculated.

Sample	- 3 min	0	+ 3 min	+ 6 min	mean	SD	RSDr
1	89	90	89	91	90	1.0	1.1%
2	143	152	145	149	147	4.3	2.9%
3	309	321	315	313	315	5.2	1.7%

3.4.3- Influence of pipetting volumes

The OkaTest assay consists of three pipetting steps of relatively small volumes. First, 50 µL samples of standards are applied in duplicate and 70 µl of phosphatase is added. Then, after the first incubation, 80 µL of substrate and finally 70 µL of stopping solution are added. The influence of pipetting error was evaluated by introducing a 2 µL systematic error in each of the pipetting steps, e.g. a -2 µL error means pipetting 48, 68, 78 and 68 µL for samples/standard, phosphatase, substrate and stopping solution, respectively. This relatively big error (4% of the sample volume) is quite above the systematic error that can be expected in correctly calibrated pipettes (2%), but it was chosen in order to get clear results for obvious interpretation. The RSDr and error were evaluated (Table 8).

Table 8. Effect of the sistematic pipetting error on the results of the test. Mean, standard deviation (SD), relative standard deviation (RSDr) and error (Errormax) were calculated.

Sample	- 2 uL	0	+ 2 uL	mean	SD	RSDr	Errormax*
1	83	85	93	87	4.9	5.6%	8.0 (9.4%)
2	161	148	156	155	6.7	4.3%	13 (8.8%)
3	303	289	304	299	8.5	2.8%	15 (5.1%)

*Errormax = maximum difference from standard (0) conditions in µg/kg and percentage.

The RSDr was at highest 5.6% and in accordance with the values normally obtained with OkaTest. The error introduced changed from 9.4 to 5.1% of the standard conditions.

The effect of a single pipetting error was evaluated by introducing a 5 µL error in one of the pipetting steps. In this case, the standard curve was performed according the kits' instructions and the error was introduced in the samples that were quantified. For example, a -5 µL error in the phosphatase means that 65 µL phosphatase was added to 50 µL sample (in duplicate) after which the assay was performed as usual. Also in this case, a relatively big error was chosen (10-6.3 % error, depending on the assay volume) (Table 9).

Table 9. Effect of a single pipetting error on the results of the test. Mean, standard deviation (SD), relative standard deviation (RSDr) and error (Errormax) were calculated.

Variable	-5 µL	0	+ 5 µL	mean	SD	RSDr	Errormax*
Sample	132	148	173	151	20.5	13.6%	25 (17%)
Phosphatase	180	148	130	153	25.2	16.5%	32 (22%)
Substrate	167	148	159	158	9.6	6.1%	19 (13%)
Stop solution	170	148	153	157	11.6	7.4%	22 (15%)

*Errormax = maximum difference from standard (0) conditions in µg/kg and percentage.

Table 9 shows that pipetting errors in sample and phosphatase volume have the biggest effect and special care have to be taken when applying these. Also the logical tendencies can be seen; when applying less samples underestimation can be expected, while with the phosphatase occurs the contrary. This is to be expected, less phosphatase means more inhibitor per amount of phosphatase and so higher estimates of the toxin concentration. Table 9 also shows that high RSDr values (above 10%, ZEU-INMUNOTEC in-house 5%) are a good indication for pipetting error.

Substrate and stop solution pipetting errors seem to be much less important RSDr < 10%).

3.4.4- Influence of phosphatase solubility

In the previous paragraph was shown that the amount of phosphatase added to each well is important for correct quantification. The phosphatase is the only component of the kit that is not ready to use. It has to be dissolved previously and insufficient solubilisation could lead to overestimation of the toxin concentration. Therefore the solubilisation time was evaluated by dissolving three phosphatase vials of the same batch for 30, 60 and 90 minutes (normal resuspension time use is 60 minutes), and

always under agitation. Three control samples were quantified and the RSDr was evaluated (table 10).

Table 10. Test results after dissolving the phosphatase for 30, the normal 60 and 90 minutes. The remaining part of the assay was performed according to the kits instructions. Mean, standard deviation (SD), relative standard deviation (RSDr) were calculated.

Sample	30 min	60 min	90 min	Mean	SD	RSDr
1	100	95	99	98	2.5	2.5%
2	167	151	157	158	8.0	5.0%
3	317	304	318	313	8.1	2.6%

The RSDr values obtained were at highest 5.0% and comparable to those obtained for within batch variability (see table 3).

3.5- Limit of detection (LOD) and limit of Quantification (LOQ)

To estimate the LOD and LOQ a blank mussel material was extracted ten times and analyzed according the kits' instructions. The mean and standard deviation were calculated and the limit of detection was estimated by the equation below:

$$LOD_{99\%} = X + 3SD$$

The LOQ (the lowest concentration that can be determined with an acceptable level of repeatability precision and trueness) was estimated using the same data and equation, but applying a higher factor:

$$LOQ_{99\%} = X + 10SD$$

The mean result obtained for the blank sample was 38 µg/kg. The estimated LOD and LOQ were 44 µg/kg and 56 µg/kg, respectively (Table 11). It is very important to observe that the LOD and LOQ are below the working range as this permits correct quantification along the complete working range of the test.

Table 11. Quantification of the standard solvent (10 repetitions) as OA concentration equivalents (µg/kg) to estimate the LOD and LOQ. Mean, standard deviation (SD), relative standard deviation (RSDr) and the repeatability limit (r) were calculated.

Repetition	Concentration OA (µg/kg)
1	36
2	38
3	36
4	37
5	41
6	37
7	40
8	38
9	40
10	38
mean	38
SD	1.8
RSDr	4.6%
LOD	44
LOQ	56
r	5.1

3.6- Repeatability

To get an idea regarding the repeatability characteristics of the assay two different mussel samples at different concentrations were tested. Samples were prepared and tested according to the kits´ instructions.

Table 12. Repeatability of 2 different mussel samples.Mean, standard deviation (SD), relative standard deviation (RSDr) and repeatability limit (r) were calculated.

repetition	sample 1 (µg/kg)	sample 2 (µg/kg)
1	269	124
2	276	125
3	276	131
4	273	129
5	280	121
6	278	117
7	281	127
8	275	118
Mean	276	124
SD	3.9	4.8
RSDr	1.4%	3.9%
r	11	14

For two of the three samples mean and relative standard deviation were calculated. The RSDr obtained for the samples tested by the kit were, 1.4 and 3.9%, respectively.

These values are far below the reference value of 15% (Horwitz, W., AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals). For sample 3 no calculations could be made as it didn´t contain sufficient OA (or its derivatives) to be detected. It is shown to demonstrate the consistency of the negative results.

3.7- Intermediate precision

The intermediate precision of the test was estimated by repeating 10 different samples on 3 different days by the same analyst. The assay was performed according to the kits instructions. For all samples the mean values and reproducibility limits were calculated (Table 13).

Table 13. Reproducibility of ten different mussel and scallops samples. Mean, standard deviation (SD), relative standard deviation (RSDr) were calculated. <63: below the working range of the assay (63 – 352 µg/kg)

sample	origin	day 1 (µg/kg)	day 2 (µg/kg)	day 3 (µg/kg)	mean	SD	RSDr
1	Mussel	211	227	187	208	19.84	9.5%
2	Mussel	122	132	113	122	9.57	7.8%
3	Scallop	<63	<63	<63	-	-	-
4	Mussel	82	94	90	88	6.17	7.0%
5	Mussel	196	196	215	202	10.57	5.2%
6	Scallop	<63	<63	<63	-	-	-
7	Mussel	<63	<63	<63	-	-	-
8	Scallop	125	108	117	117	8.20	7.0%
9	Mussel	250	253	281	261	16.90	6.5%
10	Mussel	277	279	289	282	6.62	2.4%

The RSDr was compared to the theoretical RSDr as calculated by Horwitz (AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals) which is about 15% for samples of this concentration range. All values, are far below this reference value. All negative samples obtained results below the working range (<63) during the three days.

3.8- Selectivity

The selectivity of the method was estimated by calculating recoveries of okadaic acid, DTX-1 and DTX-2 and finally by testing a certified reference material.

Certified DTX1 and DTX2 materials were not available at the time this validation was first performed Additional assays with certified DTX1 and DTX 2 to confirm the results obtained showed in this report are currently being carried out and they will provide them shortly.

3.8.1- OA

The selectivity of the assay was estimated by preparing 20 tubes with 5 ± 0.1 g of a mussel market sample, containing 90 µg /kg of OA, and spiking 5 of these tubes with 0, 80, 160 or 240 µg/kg of okadaic acid (CRM-OA-c, NRC-CNRC, Marine Analytical Chemistry Standards Program. Institute for Marine Biosciences). The spiked samples were analysed according to the kits´ instructions on three separate days. Repetition 1 was analysed on the first day, repetitions 2 and 3 on the second day while repetitions 4 and 5 were analysed on a third day (Table 14).

Table 14. Recoveries of OA spiked at different concentrations on mussel. Mean, standard deviation (SD), relative standard deviation (RSDr) and recovery were calculated. Market sample used for spiking contained 90 µg OA/kg before spiking.

mussel sample	**spiked OA (µg/kg)**			
repetition	**0**	**80**	**160**	**240**
1	86	158	230	271
2	87	134	211	282
3	87	178	216	257
4	95	193	253	298
5	95	191	257	280
Mean	90	171	233	277
SD	4.8	25.0	20.9	15.1
RSDr	5.4%	14.6%	8.9%	5.4%
Recovery		101%	90%	78%

The mean OA content of the 5 sample preparations was calculated and used to estimate the recovery that ranged from 78 % to 101 % (table 14). These recoveries are in agreement with the 70 to 125% range (Horwitz., AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals) that is expected for this concentration range although there could be a tendency for underestimating the amount of OA at higher concentrations.

The RSDr for all repetitions was below 15% the expected value under repeatability conditions at these concentration even though the experiment was performed under more demanding circumstances (different days, independently spiked samples).

To get an idea of the matrix effect on toxin recovery the same experiment was performed with king scallop. A market sample was acquired, prepared and analysed according the kits´ instructions. Also for this experiment the assays were performed on different days. Repetition 1 was analysed on the first day, repetitions 2 and 3 on the second day while repetitions 4 and 5 were analysed on a third day (Table 15).

Table 15. Recoveries of OA spiked at different concentrations on king scallop. Mean, standard deviation (SD), relative standard deviation (RSDr) and recovery were calculated. ND: none detected.

king scallop sample	spiked OA (µg/kg)			
repetition	0	80	160	240
1	ND	82	162	252
2	ND	84	142	218
3	ND	89	150	268
4	ND	102	177	268
5	ND	99	158	271
Mean	0	91	157	255
SD		9.0	13.3	22.2
RSDr		9.9%	8.4%	8.7%
Recovery		114%	98%	106%

The mean OA content of the 5 sample preparations was calculated and used to estimate the recovery that ranged from 98 % to 114 % (table 15). These recoveries are in agreement with the 80 to 110% range that is expected for this concentration range although there could be a tendency for overestimating the amount of OA at lower concentrations. Also here the RSDr for all repetitions was below the expected 15% (see above).

3.8.2- Dinophysistoxins 1 and 2 (DTX-1 and DTX-2)

All the okadaic acid group toxins that are described at this moment can be transformed by hydrolysis to OA, DTX-1 or DTX-2 all of which can be detected by the phosphatase (see also 3.9 applicability). In order to verify this, a king scallop sample was prepared according the kits´ instructions and 5 g aliquotes were spiked with 0, 80, 160 or 240 µg/kg of DTX-1 (Wako Pure Chemical Industries, Ltd.) Also for this experiment the assays were performed on different days. Repetition 1 was analysed on the first day,

repetitions 2 and 3 on the second day while repetitions 4 and 5 were analysed on a third day (Table 16).

The mean DTX-1 content of the 5 sample preparations was calculated and used to estimate the recovery, that ranged from 79 % to 102 % (table 16). These recoveries are in agreement with the 80 to 110% range that is expected for this concentration range. In general higher RSDr values that are observed for this experiment seem to be higher and at 240 µg/kg it was above the 15% limit for repeatability. However, the difference is small (16.9% compared to 15%) and can be expected in the view of the extra sources of variability that were part of this experiment (different days, independently spiked aliquotes).

Table 16. Recoveries of DTX-1 spiked at different concentrations on king scallop. Mean, standard deviation (SD), relative standard deviation (RSDr) and recovery were calculated. ND: none detected.

king scallop sample	spiked DTX - 1 (µg/kg)			
repetition	**0**	**80**	**160**	**240**
1	ND	63	101	211
2	ND	91	127	179
3	ND	81	132	175
4	ND	82	132	261
5	ND	93	140	228
Mean	0	82	126	211
SD		11.9	14.8	35.6
RSDr		14.5%	11.7%	16.9%
Recovery		102%	79%	88%

For DTX-2 (Laboratorio Cifga S.A.) a similar, but smaller scale experiment was designed. Only two aliquotes of a mussel sample were spiked at 80 µg/kg due to lack of toxin. The samples were analysed in the same assay (Table 17).

Table 17. Recoveries of DTX-2 spiked at different concentrations on mussel. Mean, standard deviation (SD), relative standard deviation (RSDr) and recovery were calculated.

mussel sample	spiked DTX-2 (µg/kg)	
repetition	**0**	**80**
1	86	157
2		163
Mean	86	160
SD	-	3.6
RSDr	-	2.3%
Recovery	-	93%

The mean DTX-2 content of the 2 sample preparations was calculated and used to estimate the recovery (93%, table 17). The recovery is in agreement with the 80 to 110% range that is expected for this concentration range. The RSDr value calculated doesn´t contain any reliable information (only 2 repetitions), but the differences between the two repetitions are small (6 µg/kg with a mean result of 160 µg/kg).

3.8.3- Spiking with certified reference material

A certified reference material is available (CRM-DSP-MUS-b; NRC-CNRC, Marine Analytical Chemistry Standards Program. Institute for Marine Biosciences). However, the certified concentration is far above the working range of the assay. Instead of diluting after extraction, a mussel market sample was acquired and homogenized for spiking. Spiking with the certified mussel material is a very delicate procedure. In order to prevent errors due to false addition of the spiking material, the amount of reference material was added, as precise as possible, to 50 mL tubes and weighed. Then, the mussel sample was added and also weighed. Finally, the amount of mussel reference material per amount of mussel market sample was calculated. This value was used as the theoretically spiked amount.

Four aliquotes were prepared and analysed according to the kits´ instructions, with the difference that the samples were also analysed without hydrolysis (table 18). This was done so because the material is only certified for OA and DTX-1 not for OA ester derivates.

Table 18. Recovery of DSP toxins from mussel sample spiked with certified reference mussel tissue. Spiked OA: theoretically spiked amount, no hydr: sample analysed without hydrolysis, hydr: sample analysed with hydrolysis.

mussel sample repetition	spiked OA (µg/kg)	no hydr OA (nM)	hydr OA (nM)	recovery no hydr	recovery hydr
1	202	142	257	71%	127%
2	189	162	305	86%	161%
3	213	204	339	96%	159%
4	270	263	364	97%	135%
			Mean	87%	146%
			SD	12.4%	17.2%
			RSDr	14.1%	11.8%

The mean OA content of the 4 sample preparations was calculated. Both the DTX-1 and OA content of the material were used to estimate the recovery. The recovery for the non hydrolysed samples ranged from 71% to 98%, with a mean 87%. These are acceptable recoveries and in accordance with the former results (paragraph 3.8.1 and 3.8.2). However, the recovery for the hydrolysed samples ranged from 127% to 159% with a mean recovery of 146% of the hydrolysed samples, far above the expected result. This means that there could be OA esters present in the sample as the material is not certified for these compounds.

The results obtained when spiking and analysing king scallop material in a similar manner (table 19) only affirm that conclusion.

Table 19. Recovery of DSP toxins from king scallop sample spiked wiht certified reference mussel tissue. Spiked OA: theoretically spiked amount, no hydr: sample analysed without hydrolysis, hydr: sample analysed with hydrolysis.

king scallop sample repetition	spiked OA (µg/kg)	no hydr OA (nM)	hydr OA (nM)	recovery no hydr	recovery hydr
1	196	186	319	95%	163%
2	148	126	233	85%	157%
3	183	167	307	91%	168%
4	191	179	316	94%	166%
			Mean	91%	163%
			SD	4.5%	4.5%
			RSDr	5.0%	2.8%

3.9- Applicability

There is an amply description in the scientific literature for the use of the protein phosphatase 2A, and different applications based on the recognition of the DSP toxins by this enzyme have been developed and patented (Tubaro et al, 1996; Honkanen et al, 1996, Vyeites eta l, 1997; Nunez et al 1997, Ramstad et al, 2001 Mounfort 2001).

Three of the toxins described to inhibit the phosphatase enzyme are commercially available and were used to determine their IC50 (concentration of toxin necessary for 50% enzyme inhibition) (Table 20). Even though differences are to be expected with regards to the sensitivities and activity of the enzyme, these depend highly on the enzyme preparation and the buffer systems used, but are expected to be within the same range.

IC50 values will be re-calculated for DTX1 and DTX2 using certified reference materials as these were not available when this study was first performed. Cross-

reactivity will also be tested in-house using yessotoxin and azaspiracids as these toxins were not commercially available at the time this study was first carried out. This data will be provided shortly

Table 20. In-house IC50 values for the OkaTest assay calculated for okadaic acid (OA), dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2) and pectenotoxin 2 (PTX-2) from standard curves obtained with three different phosphatase batches.

	IC50 (nM)					
Toxin	**Batch 1**	**Batch 2**	**Batch 3**	**mean**	**SD**	**RSDr**
OA	1.2	1.3	1.1	1.2	0.10	8.2%
DTX-1	1.6	1.6	1.5	1.6	0.08	5.1%
DTX-2	1.1	1.2	1.2	1.2	0.06	4.9%
PTX-2	>100	>100	>100	-	-	-

The IC50 values are very similar for all three toxins tested, being 1.2 nM for OA and DTX-2, and 1.6 nM for DTX-1. The latter showing a slightly lower toxicity. This can also be seen in the inhibition curves (fig. 3) where the curves for OA and DTX-2 are completely overlapping while the curve for DTX-1 has a lower slope. This result could lead to lower recoveries. However, the recovery experiments performed (see 3.8.2) didn´t show that behaviour.

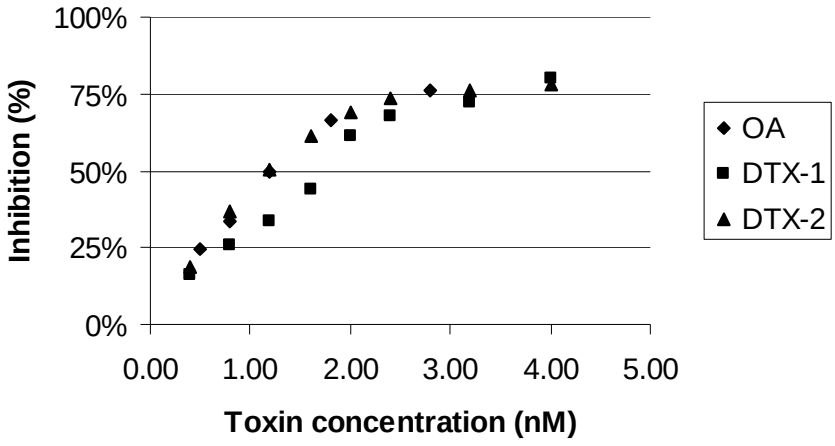


Fig. 3. Phosphatase inhibition curve obtained with okadaic acid (OA), dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2).

The IC_{50} values found in our study are in accordance with the ones obtained recently by Huhn et al., 2009. However, these do not exactly correspond to the toxicity factors

(TEF) that are used in analytical methods such as LC-MS/MS; as OA and DTX-1 have a TEF of 1, while DTX-2 has a TEF of 0.6, indicating equal toxicity for DTX-1 and OA and less toxicity for DTX-2 (EFSA, 2008).

3.10. Measurement of Uncertainty

Measurement of Uncertainty was calculated according to Horwitz W, 2002. Reproducibility data from 5 different samples obtained in a collaborative study (Smienk et al, 2013) was used to calculate the expanded uncertainty (Table 21).

Table 21: Expanded uncertainty (U) was calculated using SR values from 5 different samples. u: uncertainty. U: Expanded uncertainty = u x 2. (1)mean µg total equiv. OA/Kg obtained from 13/15 labs (depending on the sample).

Sample	Mean(1) µg total equiv. OA/Kg	S_R	RSD_R (%)
Clam *(Venerupis pollastra(*	242.8	19,4	8
Mussel *(Mytilus galloprovincialis)*	98.8	10,7	10,7
Clam *(Venerupis decussatus)*	275	21	7,6
Cockle *(Cerastoderma edulis)*	175.4	23,2	13,2
Mussel *(Mytilus edulis)*	255	20,7	8,1
	u	19,0	9,5
	U	**38,0**	**19,0**

3.11- Method comparison

A method comparison was performed with the mouse bioassay (MBA) and LC-MS/MS.

To compare results from OkaTest to MBA, samples by OkaTest with a concentration ≥ 160 µg/kg were regarded as positive while samples with a concentration < 160 µg/kg were reported negative.

A total of thirtyone samples were tested by MBA, OkaTest and LC-MS/MS. Twentythree of these samples tested positive for both methods and five samples tested negative for both methods. However, three samples were positive for MBA and negative for OkaTest (Table 22). In all three samples OA toxins were detected, but below the regulatory limit of 160 µg/kg (144, 135 and 124 µg/kg OA toxins, respectively). Those samples were tested by LC-MS/MS coincided with MBA on two

out of three results, showing and showing slightly higher quantifications compared to OkaTest (185, 152 and 177 OA toxins µg/kg, respectively).

Table 22. Results from MBA, OkaTest and LC-MS/MS. Positive results (+): ≥160 µk/kg. Negative result (-): <160 µg/kg.

Matrix	MBA	OkaTest	OkaTest µg OA equiv. /kg	LC-MS/MS µg OA. /kg
Mussel	-	-	122	ND
Scallop	-	-	ND	ND
Mussel	-	-	ND	ND
Donax	-	-	97	82
Cockle	-	-	ND	ND
Mussel	+	+	196	158
Mussel	+	+	232	502
Mussel	+	+	268	ND
Scallop	+	+	264	184
Mussel	+	+	250	177
Mussel	+	+	265	288
Mussel	+	+	196	318
Mussel	+	+	>377	604
Mussel	+	+	>377	894
Mussel	+	+	277	390
Mussel	+	+	305	658
Mussel	+	+	306	414
Mussel	+	+	310	392
Mussel	+	+	>377	444
Mussel	+	+	315	329
Mussel	+	+	270	232
Mussel	+	+	277	235
Mussel	+	-	135	152
Mussel	+	+	164	98
Mussel	+	+	211	168
Mussel	+	+	251	209
Mussel	+	+	191	113
Mussel	+	-	124	177
Cockle	+	+	252	193
Mussel	+	+	216	247
Mussel	+	-	144	185
Mussel		-	ND	ND
Mussel		+	>377	357
Mussel		-	ND	292
Mussel		-	ND	ND
Mussel		-	ND	ND
Mussel		+	304	316

* HPLC-MS results were not with toxicity factors. However only 4 samples contained DTX-2.

Thirtytwo samples from clams, mussles and oysters from the United States were also tested by OkaTest and EU harmonized LC-MS/MS (version 2, 2010) (Table 23).

Table 23. Results from OkaTest and LC-MS/MS (EU harmonized SOP, v2, 2010)
Assays carried out by Jonathan Deeds from the FDA, US

Shellfish/Location	OkaTest (µg equiv. OA/Kg)	LC-MS/MS			
		OA	DTX1	DTX2	Total µg equiv. OA/Kg
Softshell Clams (Mya arenaria) State: New York US East Coast Atlantic Ocean	336	255	118	ND	373
	315	202	105	ND	325
	295	217	108	ND	307
	285	136	69	ND	260
	240	171	89	ND	205
	190	102	53	ND	155
	118	49	26	ND	75
	<63	26	13	ND	39
	<63	ND	ND	ND	ND
Oysters (Crassostrea virginica) State: Texas US Gulf Coast Gulf of Mexico	322	563	ND	ND	563
	300	519	ND	ND	519
	245	202	ND	ND	202
	240	194	ND	ND	194
	239	221	ND	ND	221
	235	189	ND	ND	189
	198	189	ND	ND	189
	155	88	ND	ND	88
	154	97	ND	ND	97
	88	38	ND	ND	38
	<63	16	ND	ND	16
Mussels (Mytilus edulis) State: Washington US West Coast Pacific Ocean	>352	ND	525	ND	525
	266	ND	272	ND	272
	256	ND	263	ND	263
	171	ND	165	ND	165
	157	ND	164	ND	164
	141	ND	131	ND	131
	134	ND	128	ND	128
	127	ND	121	ND	121
	90	ND	76	ND	76
	<63	ND	76	ND	76
	<63	ND	33	ND	33
	<63	ND	ND	ND	ND

4- LITERATURE

- EFSA. *Marine biotoxins in shellfish – okadaic acid and analogues. Scientific Opinion of the Panel on Contaminants in the Food chain*. Scientific Opinion of the Panel on Contaminants in the Food chain. The EFSA J., 589, 1-62, 2008

- *EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS*, version 2, July 2010.

- Honkanen RE, Mowdy DE, Dickey RW. *Detection of DSP-toxins, okadaic acid, and dinophysis toxin-1 in shellfish by serine/threonine protein phosphatase assay*. J. AOAC Int. Nov-Dec;79 (6):1336-43, 1996.

- Huhn, J.; Jeffrey, P.D.; Larsen, K.; Rundberget, T.; Rise, F.; Cox, N.R.; Arcus, V.; Shi, Y.; Miles C.O. *A structural basis for the reduced toxicity of dinophysistoxin-2*. Chem. Res. Toxicol., 22, 1782-1786**,** 2009

- Mounfort D. O. et al. *Protein phosphatase inhibition assay adapted for determination of total DSP in contaminated mussels.* Toxicon 39, pp 383-390, 2001.

- Nunez PE; Scoging AC; *Comparison of a protein phosphatase inhibition assay, HPLC assay and enzyme-linked immunosorbent assay with the mouse bioassay for the detection of diarrhetic shellfish poisoning toxins in European shellfish*. Int J Food Microbiol. Apr 29; 36 (1):39-48, 1997.

- Ramstad H, Shen JL, Larsen S, Aune Te, *The validity of two HPLC methods and a colorimetric PP2A assay related to the mouse bioassay in quantification of diarrhetic toxins in blue mussels (Mytilus edulis).*Toxicon. Sep;39 (9):1387-91, 2001.

- Smienk H., Domínguez E., Rodríguez-Velasco M.L. Clarke D., Katrin K., Katikou P., Cabado A.G., Otero A., Vieties J.M. Razquin P., and Mata L. *Quantitative Determination of the Okadaic Acid Toxins Group by a Colorimetric Phosphatase Inhibition Assay: Interlaboratory Study. Journal AOAC*, 96, 1 , 77-85, 2013.

- Takai A and Mieskes. *Inhibitory effect of okadaic acid on the p-nitrophenyl phosphate phosphatase activity of protein phosphatases.*, Biochem. J. 275, pp 233-239, 1991.

- Tubaro A, Florio C, Luxich E, Sosa S, Della Loggia R, Yasumoto T. *A protein phosphatase 2A inhibition assay for a fast and sensitive assessment of okadaic acid contamination in mussels.* Toxicon., Jul;34 (7):743-52, 1996.

- Vieytes M.R., Fontal O.I., Leira F., Baptista de Sousa J.M.V., and Botana L.M. *A Fluorescente microplate assay for diarrheic shellfish toxins.* Analytical Biochemistry, 248, pp 258-264, 1997.

- William Horwitz, *Protocol for the design, conduct and interpretation of method-performance studies.* Pure & Appl. Chem., Vol 67, No. 2, pp 331-343, 1995.

- William Horwitz, AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals. 12-19, 2002.

5- APPENDIX I-

Symbols and terms for designating parameters developed by a method-performance study.

Standard Deviation…….……	***SD***
Relative Standard Deviation……..	***RSD***
r……	***Repeatability limit***
R……	***Reproducibility limit***

.

x = EXP (y - b/a)

Where x is the OA concentration in the sample (Cs) and y the absorbance of the sample.

Note: An Excel worksheet to calculate results is available upon request.

3.- Calculate the diarrheic shellfish toxins concentration in tissue (Ct) as follows:

$$Ct\ (\mu g/kg) = \frac{(Cs\ (nM) \times FD \times MW\ (g/mol) \times Ve\ (L))}{Mt\ (g)}$$

Ct: toxins concentration in tissue, expressed as equivalents of OA; Cs: toxins concentration in sample; FD: Methanolic extract dilution factor (i.e. 640 µL/20 mL → x 31.25); MW: Okadaic acid molecular weight = 805; Ve: Methanolic extract volume (0.025L); Mt: Tissue weight (5g).

Example: for OA concentration of 1.5 nM: 1.5 nM x 31.25 x 805 g/mol x 0.025L / 5g = 189 µg OA q/kg.
For samples with OA concentration falling outside the working range (< 0.5 nM or > 2.8 nM), results will be reported as < 0.5 nM (or < 63 µg/Kg) or > 2.8 nM (or > 352 µg/kg), respectively.

F. Importance of Okadaic Acid Determination

Okadaic Acid is one of the "diarrheic shellfish poisons" (DSP) produced by the dinoflagellate species *Dinophysis* and *Prorocentrum*. Contamination of shellfish with okadaic acid has been associated with harmful algal blooms throughout the world.

In man, DSP causes dose-dependent symptoms of diarrhea, nausea, and vomiting. The action level established by the FDA is 0.2ppm. The EU has established a level of 160ug OA eq (OA, DTXs, PTXs)/kg.

The Okadaic Acid Phosphattase assay allows the determination of 40 samples in duplicate determination. Only a few milliliters of sample are required. The test can be performed in less than 1 hour.

G. REFERENCES

1. A Fluorescent Microplate Assay for Diarrheic Shellfish Toxins. Vieytes M. R. et al. Analytical Biochemistry 248, 258-264 (1997).
2. Protein phosphatase inhibition assay adapted for determination of total DSP in contaminated mussels. Mounfort D. O. et al. Toxicon 39, 383-390 (2001).
3. Inter-laboratory validation of the fluorescent protein phosphatase inhibition assay to determine diarrheic shellfish toxins: intercomparison with liquid chromatography and mouse bioassay. Gonzalez J. C., et al. Analytica Chimica Acta 466, 233-246 (2002).

For ordering or technical assistance contact:

Abraxis LLC
54 Steamwhistle Drive
Warminster, PA 18974
Tel.: (215) 357-3911
Fax: (215) 357-5232
Email: info@abraxiskits.com
WEB: www.abraxiskits.com

Kit reagents are manufactured by Zeu-Immunotec.

012412

Okadaic Acid (PP2A), Microtiter Plate

Test for the Detection of Okadaic Acid-toxins group

Product No. 520025

1. General Description

This protocol specifies a method for the quantitative determination of Okadaic Acid (OA) and other carboxylic toxins of the OA group including DTX1, DTX2 and DTX3 by a colorimetric phosphatase inhibition assay. This method is applicable to shellfish species such as mussels, clams, oysters and scallops.

2. Safety Instructions

The standard solutions in this test kit contain small amounts of Okadaic Acid in solution. Avoid contact of standard and stopping solutions with skin and mucous membranes. If these reagents come in contact with the skin, wash with water. Recommended: Polypropylene material should be avoided throughout sample collection, conservation and treatment, since loss of toxins has been shown to occur.

3. Storage and Stability

The Okadaic Acid-PP2A Kit should to be stored in the refrigerator (4–8°C) prior to use and protected from light. The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the expiration date on the box.

4. Test Principle

Test based on the phophatase activity inhibition by OA-toxins group, responsible for diarrheic shellfish poisoning (DSP). Phosphatase enzyme PP2A is able to hydrolyse a specific substrate, yielding a product that can be detected at 405 nm. Samples containing toxins from the okadaic acid group will inhibit the enzyme activity proportionally to the amount of toxin contained in the sample. The concentration of toxin in the sample can be calculated using a standard curve.

5. Limitations of the Okadaic Phosphatase Assay, Possible Test Interference

Numerous organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in water samples, test interferences caused by matrix effects can't be completely excluded.

Mistakes in handling the test can also cause errors. Possible sources for such errors can be:

Inadequate storage conditions of the test kit, wrong pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the assay and/or substrate reaction, extreme temperatures during the test performance (lower than 10°C or higher than 40°C). The assay procedure should be performed away from direct sun light.

As with any analytical technique (GC, HPLC, mouse bioassay, etc…..) positive results requiring some action should be confirmed by an alternative method.

Working Instuctions

A. Materials Provided

1. Microtiter plate
2. Phosphatase, 4 vials
3. Standards Okadaic Acid (5): 0.5, 0.8, 1.2, 1.8, and 2.8 nM
4. Chromogenic Substrate, 1 vial
5. Phosphatase Dilution Buffer, 1 vial
6. Stock Buffer Solution, 1 vial
7. Stop Soultion, 1 vial
8. Adhesive Film

B. Additional Materials (not included with the test kit)

1. Micro-pipettes with disposable plastic tips (10-200 and 200-1000 µL)
2. Multi-channel pipette (50-250 µL) or stepper pipette with plastic tips (10-250 µL)
3. Microtiter plate reader (wave length 405 nm)
4. Timer
5. Tape or Parafilm
6. Glass vials with Teflon-lined caps
7. Distilled or deionized water
8. Vortex mixer
9. Heater at 30 +/- 2 °C
10. Water bath at 76 +/- 2 °C
11. Methanol (analytical grade)
12. NaOH, 2.5 N (analytical grade)
13. HCl, 2.5N (analytical grade)
14. Deionized water (grade 2, ISO3696)
15. Graded 50 mL centrifuge tubes with screw caps
16. Tube shaker

C. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel pipette or a stepping pipette for adding the assay buffer, substrate and stop solutions in order to equalize the incubations periods of the solutions on the entire microtiter plate. Please use only the reagents and standards from one package lot in one test, as they have been adjusted in combination.

SOLUTIONS

1.- Okadaic Acid Standards: to make sure these solutions are homogeneous, it is very important to mix well using a vortex, before applying to the plate.

2.- Chromogenic Substrate solution: The solution contains stabilization resin. Make sure this resin is not added to the microwells. To assure that, it is recommended to transfer the volume needed into a transparent labware (i.e.: test tube or eppendorf) and take the solution from that container to add into the wells. *Note:* Do not use this solution if the absorbance of 90 µL of this solution at 405 nm is over 0.6.

3.- Phophatase solution: Add 2.0 mL of phosphatase dilution buffer *(Phosphatase Dilution Buffer)* to one of the phosphatase vials *(Phosphatase)* and dissolve by mixing gently for 1 hour ± 5 minutes at room temperature (22 ± 2 ºC) to ensure that the enzyme is fully hydrated. Do not use the tube shaker at any moment. This solution must be stored under refrigeration if not in use immediately after preparation. Do not use the phosphatase solution for following days. Each enzyme vial contains enough volume for 24 wells. If more than one vial is used in the assay, dissolve each vial as described above, make a pool with the content of the vials and mix gently, by inversion, before use.
*Attention: this reagent is blue and becomes brownish when dissolved. If brownish colour is noticed before hydratation, discard this reagent as it could be damaged.

4.- Buffer solution x1: dilute the *Stock Buffer Solution* included in the kit by mixing 1 volume with 9 volumes of deionized water. Use buffer solution x1 only freshly made, and store under refrigeration if not in use immediately after preparation.

5.- 2.5 N NaOH: weigh 100 g of NaOH and add 500 mL of water and dissolve. Transfer to a volume-tric flask and add deionized water up to a final volume of 1000 mL.

6.- 2.5 N HCl: add 205 mL of HCl (37 %) to 400 mL of deionised water already contained in a volumetric flask. Make the volume up to 1000 mL with deionized water.

D. Assay Procedure

Warning:

The volume of some reagents used in this assay is small and special attention must be paid when added to the wells:

- Make sure the pipettes are calibrated before running the assay.
- Use pipettes according to the volumes to be dispensed. Use pipettes with a maximum pipette volume of 100 or 200 µL.
- Be sure that the incubator's temperature is stabilized before use.

It is recommended to run samples and standards in duplicate.

1.- Add 50 µL of samples or standards.
2.- Add 70 µL of the Phosphatase Solution to each well. Mix well by gentle tapping on the side of the plate.
3.- Cover the plate with the adhesive film provided and incubate for 20 ± 0.5 minutes at 30 ± 2 ºC.
4.- Remove the adhesive film and add 90 µL of Chromogenic Substrate to each well. Mix well by gently tapping on the side of the plate.
5.- Cover the plate with the adhesive film and incubate 30 ± 0.5 minutes at 30 ± 2 ºC.
6.- Remove the adhesive film and add 70 µL of Stop Solution to each well.
7.- Read absorbance of samples and standards at 405 nm.

E. Sample Preparation

The method described below includes a hydrolysis step to detect all toxins forms of okadaic acid (okadaic acid and dinophisistoxins).

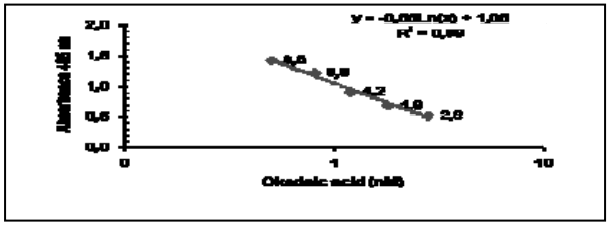
1.- Clean the shell thoroughly using water
2.- Open the shellfish by cutting the abductor muscles.
3.- Wash inside the shell thoroughly to get rid of any dirt.
4.- Remove the tissue inside the shell by cutting all the muscles attached to the shell.
5.- Place the shellfish tissue in a filter paper for few minutes to remove water in excess.

It is recommended to use graded 50 mL centrifuge tubes with screw caps during the following steps of hydrolysis in order to prevent loses due to labware changes.

6.- Mash the shellfish tissue to obtain a representative sample and weigh 5 g. Add 25 mL of Methanol and homogenise the mixture for 2 minutes using a tube shaker.
7.- Centrifuge at 2000 g for 10 min at 4 ºC. The supernatant (*methanolic extract*) is poured into a centrifuge tube.
8.- Take 640 µL of *methanolic extract* and pour into another centrifuge tube.
9.- Add 100 µL of 2.5 N NaOH.
10.- Seal and heat at 76 ± 2 ºC for 40 minutes.
11.- Add 80 µL of 2.5 N HCl (the sample does not need to be cooled down previously).
12.- Add up to 20 mL of Buffer solution x1.

E. Calculations and Graphic Representation of Results

1.- Obtain a standard curve by plotting the absorbance values in a linear *y axis* and the concentration of okadaic acid in a logarithmic *x axis* and use a logarithmic fitting as shown in the graphic next page. R^2 has to be greater than or equal to 0.96.

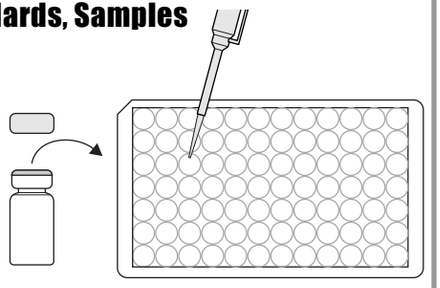


2.- The OA concentration contained in the sample (Cs) is calculated by interpolation into the calibration curve or using the following equation:

Okadaic Acid (DSP) PP2A Plate Kit, Detailed Procedure

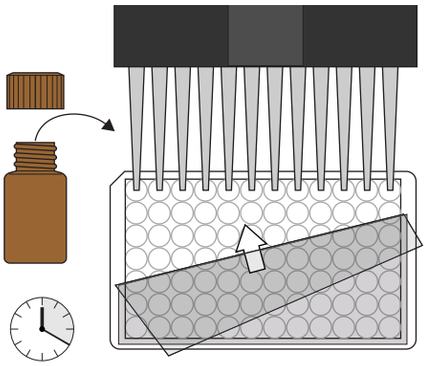
1. Addition of Standards, Samples

Add 50 uL of the standard solutions, and samples into the wells of the test strips according to the working scheme given. We recommend using duplicates or triplicates.



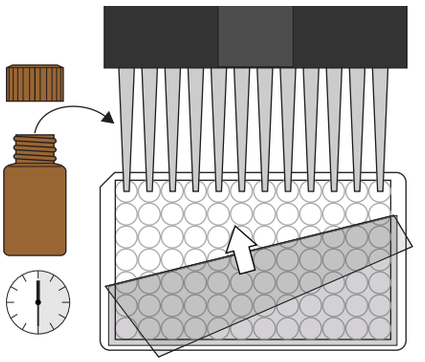
2. Addition of Phosphatase Solution

Add 70 uL of the Phosphatase solution to the individual wells successively using a multi- channel pipette or a stepping pipette. Incubate the strips for 20 min at 30ºC.



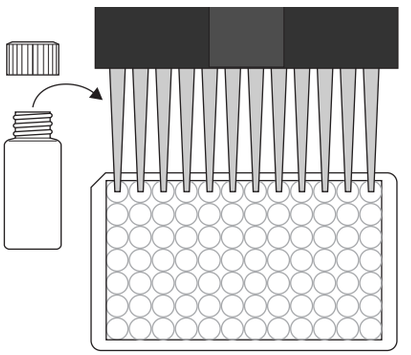
3. Addition of Chromogenic Substrate

Add 90 uL of the Chromogenic Substrate to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a rapid circular motion on the benchtop. Be careful not to spill contents. Incubate the strips for 30 min. at 30ºC.



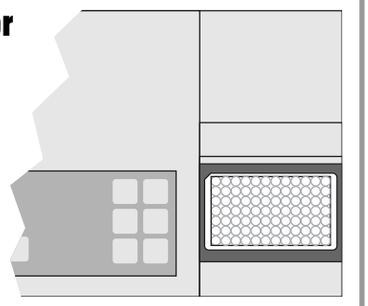
4. Addition of Stopping Solution

Add 70 uL of stop solution to the wells in the same sequence as for the substrate solution using a multi- channel pipette or a stepping pipette.



5. Measurement of Color

Read the absorbance at 405 nm using a microplate ELISA reader. Calculate results.

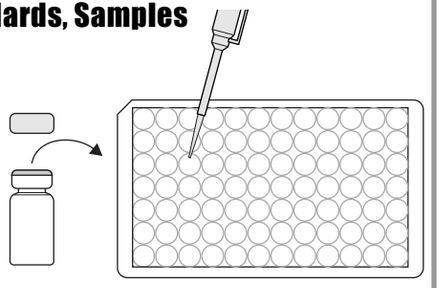


For Ordering or Technical Assistance Contact:
ABRAXIS, LLC
54 Steamwhistle Drive, Warminster, PA 18974
Phone: 215-357-3911 Fax: 215-357-5232
www.abraxiskits.com

Okadaic Acid (DSP) PP2A Plate Kit, Concise Procedure

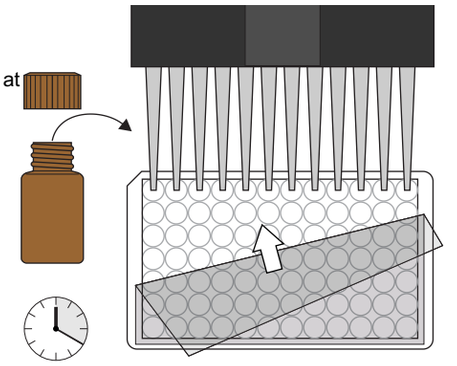
1. Addition of Standards, Samples

Add 50 uL of standard solutions, and samples.



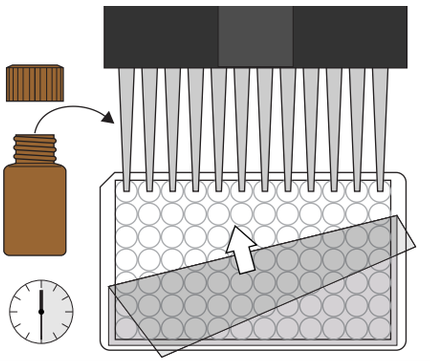
2. Addition of Phosphatase Solution

Add 70 uL of the Phosphatase solution. Incubate for 20 minutes at 30ºC.
.



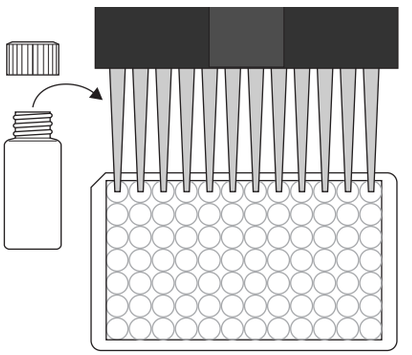
3. Addition of Chromogenic Substrate

Add 90 uL of Chromogenic Substrate. Cover and mix for 30 seconds by rotating on benchtop. Incubate for 30 minutes at 30ºC.
.



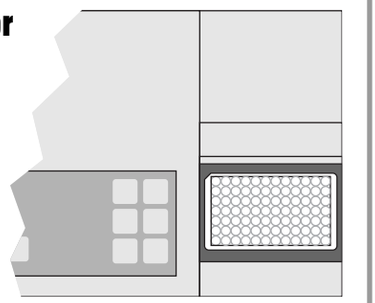
4. Addition of Stopping Solution

Add 70 uL of Stopping Solution.



5. Measurement of Color

Read the absorbance at 405 nm using a microplate ELISA reader. Calculate results.



For Ordering or Technical Assistance Contact:
ABRAXIS, LLC
54 Steamwhistle Drive, Warminster, PA 18974
Phone: 215-357-3911 Fax: 215-357-5232
www.abraxiskits.com