OKATEST

ZE/OA48C
ZE/OA96C

Test for detection of Okadaic Acid-toxins group

Test para la detección de las toxinas del grupo del Ácido Okadaico

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SCOPE

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, cockle, scallops, etc.

PRINCIPLE

Test based on the phophatase activity inhibition by OA-toxins group, responsible for diarrheic shell-fish 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.

KIT CONTENTS

	48 Tests Kit	96 Tests Kit
Microtiter plate strips (8 wells per strip)	6	12
Vials of Phosphatase *(Phosphatase)*	2	4
Set of Okadaic Acid Standards (*Okadaic acid 0.5, 0.8, 1.2, 1.8 and 2.8 nM)*	1	1
Chromogenic Substrate *(Chromogenic Substrate)*	1	1
Phosphatase Dilution Buffer *(Phosphatase Dilution Buffer)*	1	1
Stock Buffer Solution *(Stock Buffer Solution)*	1	1
Stop Solution *(Stop Solution)*	1	1
Adhesive film	1	2
Kit instructions	1	1

ADDITIONAL MATERIAL AND REAGENTS NEEDED

- Micropipettes
- Blender (Ultraturax) or mortar and pestle
- Heater at 30ºC ± 2 ºC (i.e. FX Incubator, Ref ZE/FX, from ZEULAB)
- Microplate reader (wavelength at 405 nm)
- Water bath for 76 ± 2 ºC
- Methanol (analytical grade)
- NaOH 2.5 N made by titration, (NaOH of analytical grade)
- HCl 2.5 N made by titration, (HCl of analytical grade)
- Deionised water (grade 2, ISO3696)
- Graded 50 mL centrifuge tubes with screw caps
- Tube shaker
- Centrifuge

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 deionised water. Use buffer solution x1 only freshly made, and store under refrigeration if not in use immediately.

5.- 2.5 N NaOH: weigh 100 g of NaOH and add 500 mL of water and dissolve. Transfer to a volumetric flask and add deionised 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 deionised water.

SAMPLES EXTRACTION

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 adductor muscles.
3.- Wash inside the shell thoroughly to remove 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.

TEST PROCEDURE

Warning:

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

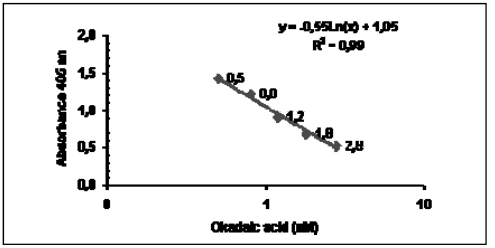
- Make sure the pipettes are calibrated before running the assay.
- Use pipettes according to the volumes to be dispensed. Use pipettes with a maximum pipette volume of 100 or 200 µL.
- Be sure that the incubator's temperature is stabilized before use.

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.

GRAPHIC REPRESENTATION AND CALCULATIONS 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:

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

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:

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

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 eq/kg.

NOTE: 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. When sample absorbance is below the value obtained for 2.8 nM the methanolic extract could be diluted up to 1:4 and samples re-tested

STABILITY AND STORAGE

The kit contents must be stored at 4 - 12 ºC and protected from light. This kit has a shelf life of 8 months when stored under optimal conditions. See the expiry date on the kit package.

SAFETY

Safety clothing should be worn and skin contact with the reagents avoided. Do not ingest.
A SAFETY DATA SHEET is available from your local distributor on request.

*Warning: Okadaic Acid is toxic. Gloves, mask and other protective clothing must be worn when handling okadaic acid solutions.

REFERENCES

1. 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.

2. Smienk H., Calvo D., Razquin P., Domínguez E. & Mata L. *Single Laboratory Validation of A Ready-to-Use Phosphatase Inhibition Assay for Detection of Okadaic Acid Toxins.* *Toxins*, 2012, *5*, 339-352.

3. 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*, 2013. 96, 1, 77-85.

OkaTest complies with the requirements established under chapter III A (4) a, b and c from Appendix III of the European Regulation (EC) 2074/2005 and can be used as complementary method.
For further information, please visit the European Reference Laboratory website:
http://aesan.msssi.gob.es/en/CRLMB/web/otros_procedimientos/other_crlmb_standard_operating_procedures.shtml

OBJETIVO

Test para la determinación cuantitativa de Ácido Okadaico (OA) y otras toxinas del grupo del OA, incluyendo DTX1, DTX2 y DTX3. Consiste en un ensayo colorimétrico de inhibición de la actividad enzimática de una fosfatasa. Este método es aplicable a especies como mejillones, almejas, berberechos, vieiras, etc.

PRINCIPIO

Okatest es un test basado en la inhibición de la actividad enzimática de una fosfatasa (PP2A) por toxinas del grupo del ácido okadaico. En condiciones normales, la fosfatasa es capaz de hidrolizar un sustrato específico obteniéndose un producto que puede ser detectado a 405 nm. En presencia de toxina diarreica se producirá una inhibición de la actividad enzimática proporcional a la cantidad de toxina diarreica presente en la muestra. Mediante la utilización de una curva de calibrado se pueden obtener los valores de concentración de toxina presentes en la muestra analizada.

COMPONENTES DEL KIT

	Kit de 48 Tests	Kit de 96 Tests
Tiras de 8 pocillos de placa microtiter	6	12
Fosfatasa *(Phosphatase)*	2	4
Set de patrones de ácido okadaico *(Okadaic acid 0.5, 0.8, 1.2, 1.8 y 2.8 nM)*	1	1
Sustrato Cromogénico *(Chromogenic Substrate)*	1	1
Solución de Dilución de la Fosfatasa *(Phosphatase Dilution Buffer)*	1	1
Solución Tamponante *(Stock Buffer Solution)*	1	1
Solución Stop *(Stop Solution)*	1	1
Lámina adhesiva	1	2
Guión de instrucciones	1	1

MATERIAL Y REACTIVOS ADICIONALES NECESARIOS

- Micropipetas
- Homogeneizador (e.j. Ultraturax) o mortero
- Incubador a 30 ± 2ºC. (Ej. FX Incubator Ref ZE/FX, de ZEULAB)
- Lector de placas microtiter con filtro a 405 nm.
- Baño termostático 76 ± 2ºC
- Metanol (grado analítico)
- NaOH (grado analítico)
- HCl (grado analítico)
- Agua desionizada (al menos de grado 2, ISO 3696)
- Tubos de centrifuga de 50 mL
- Centrífuga
- Agitador para tubos (tipo vortex)

SOLUCIONES

1.- Estándares de Ácido Okadaico: Es muy importante agitar bien estas disoluciones justo antes de su utilización (p.e.: en vortex), para asegurar su homogeneidad
2.- Sustrato Cromogénico: esta solución contiene una resina estabilizante que no debe añadirse a los pocillos. Con este fin, se recomienda transvasar el volumen a utilizar a un vial transparente (p.e.: eppendorf o tubo de ensayo), asegurándose de no coger resina, y de ahí pipetear a los pocillos. *Nota*: no usar esta solución si la absorbancia de 90 μL es superior a 0.6.
3.- Preparación de la Fosfatasa: reconstituir el liofilizado de Fosfatasa (*Phosphatase*) en 2.0 mL de Solución de Dilución de la Fosfatasa (*Phosphatase Dilution Buffer*). Mantener la solución a temperatura ambiente (22 ± 2ºC) y con agitación suave durante 1 hora para asegurar así la correcta hidratación del liofilizado. No usar el agitador de tubos en ningún momento. Una vez reconstituido el enzima, mantenerlo en condiciones de refrigeración. No conservar la solución de Fosfatasa para su uso en días posteriores.
Cada vial de Fostatasa contiene la cantidad necesaria para 24 pocillos. Si se va a utilizar más de uno, disolver cada vial como se ha explicado anteriormente y mezclar el contenido de todos en uno único antes de usar. Agitar suavemente antes de su utilización.
Atención: el liofilizado posee una coloración azulada y al reconstituirlo se convierte en marrón. Si observa que este reactivo posee una coloración marrón antes de reconstituirlo, no usarlo, ya que podría estar dañado.
4.- Solución Tamponante x1: diluir la *Stock Buffer Solution* incluida en el kit, mezclando 1 volumen de esta solución con 9 volúmenes de agua desionizada. Preparar sólo la que se vaya a utilizar en el momento y mantener en refrigeración hasta entonces.
5.- NaOH 2.5 N: pesar 100 g de NaOH y disolver en 500 mL de agua desionizada. Seguidamente, enrasar hasta un volumen final de 1000 mL usando un matraz aforado.
6.- HCl 2.5 N: Añadir 205 mL de HCl (37 %) a 400 mL de agua desionizada. Mezclar y enrasar hasta 1000 mL con agua desionizada usando un matraz aforado.

EXTRACCIÓN DE LAS MUESTRAS

El método de preparación de muestras que se describe a continuación incluye una etapa de hidrólisis que permite la detección de todas las formas tóxicas de ácido okadaico (ácido okadaico y dinofisistoxinas).

1.- Limpiar la superficie externa del molusco con agua.
2.- Abrir los moluscos seccionando los músculos aductores.
3.- Lavar el contenido de las conchas con agua hasta conseguir eliminar todas las sustancias extrañas que puedan contener.
4.- Separar la carne de las conchas, retirando todos los músculos o tejidos que estén en contacto con ellas.
5.- Colocarlos en un papel de filtro y dejarlos secar durante unos minutos.
Se recomienda el uso de tubos calibrados para centrífuga de 50 mL durante las siguientes etapas de hidrólisis para evitar pérdidas por transvase de líquidos.
6.- Triturar el tejido hasta obtener una muestra homogénea, tomar 5 g (peso húmedo) y extraer con 25 mL de Metanol durante 2 minutos, usando un agitador para tubos.
7.- Centrifugar el homogeneizado a 2000 g durante 10 minutos a 4 ºC. Al sobrenadante lo llamaremos *extracto metanólico* y lo pasaremos a otro tubo de centrífuga por decantación.

8.- Tomar 640 µL del *extracto metanólico* y transvasarlo a un tubo para centrífuga nuevo.
9.- Añadir 100 µL de NaOH 2.5 N.
10.- Cerrar y calentar la muestra a 76 ± 2 ºC durante 40 minutos.
11.- Sin dejar enfriar, añadir 80 µL de HCl 2.5 N
12.- Añadir Solución Tamponante x1 hasta un volumen final de 20 mL.

PROCEDIMIENTO DE ENSAYO

Atención:

En este ensayo se usan reactivos en volúmenes pequeños y se debe tener especial cuidado cuando se añaden a la placa:

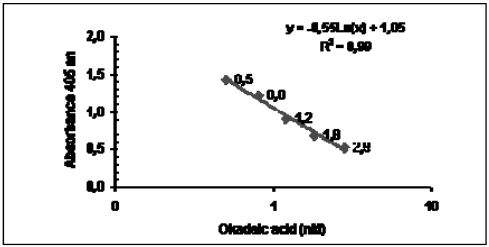
- Asegurarse de que las pipetas están calibradas antes de realizar el ensayo.
- Usar pipetas de 100 ó 200 µL de volumen máximo.
- Comprobar que la temperatura del incubador está estabilizada antes de su uso.

Es aconsejable aplicar las muestras y patrones por duplicado.

1.- Aplicar 50 µL de cada estándar o muestra.
2.- Aplicar en cada pocillo 70 µL de la Solución de Fosfatasa. Mezclar bien golpeando suavemente en el lateral de la placa.
3.- Tapar la placa con la lámina adhesiva incluida en el kit e incubar a 30 ± 2 ºC durante 20 ± 0.5 minutos.
4.- Aplicar 90 µL en cada pocillo de Sustrato Cromogénico y tapar la placa con la lámina adhesiva.
5.- Incubar a 30 ± 2 ºC durante 30 ± 0.5 minutos.
6.- Retirar la lámina adhesiva y añadir en cada pocillo 70 µL de Solución Stop.
7.– Leer la absorbancia a 405 nm en un lector de placas microtiter.

REPRESENTACIÓN Y CÁLCULO DE LOS RESULTADOS

1.- Obtener una curva de calibrado representando las absorbancias en el eje de ordenadas frente a las concentraciones de ácido okadaico en el eje de abscisas (este último en escala logarítmica). A continuación se muestra un ejemplo de curva patrón. R^2 deberá ser mayor o igual a 0.96.



2.- A partir de la curva de calibrado obtener los valores de ácido okadaico de las muestras (Cs) por interpolación o aplicando la ecuación correspondiente:

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

x: concentración de ácido okadaico en la muestra
y: absorbancia de la muestra

*ZEULAB puede proporcionar una plantilla Excel para calcular los resultados. Para más información contacte con nosotros.

3.- Calcular la concentración de toxinas diarreicas en el tejido (Ct) a partir de la siguiente fórmula:

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

Ct: Concentración de toxinas en tejido; Cs: Concentración de toxinas de cada muestra aplicada en el pocillo; FD: Factor de dilución del extracto metanólico en la preparación de la muestra (p.e. 640 µL/20 mL → x 31.25); PM: Peso molecular ácido okadaico = 805; Ve: Volumen de extracto metanólico obtenido (0.025L); Mt: Masa de tejido pesada inicialmente (5 g).

Ej.: Para una muestra 1.5 nM de OA: 1.5 nM x 31.25 x 805 g/mol x 0.025 L / 5 g = 189 µg eq OA/kg

NOTA: Aquellas muestras cuya concentración (Cs) esté fuera del rango de trabajo (< 0.5 nM ó > 2.8 nM), los resultados se expresarán como < 0.5 nM (ó < 63 µg/Kg) ó > 2.8 nM (ó > 352 µg/kg) respectivamente.
Muestras con absorbancias inferiores a las obtenidas para el patrón 2.8 nM pueden ser analizadas de nuevo haciendo una dilución máxima de1:4 del extracto metanólico.

ESTABILIDAD Y ALMACENAMIENTO

Conservar los componentes del kit de 4 -12 ºC y en oscuridad. El kit tiene una estabilidad de 8 meses en las condiciones de conservación anteriormente indicadas.

SEGURIDAD

Se recomienda seguir unas prácticas correctas de laboratorio, así como el empleo de ropa y material de seguridad adecuados para el desarrollo del test. Evitar el contacto directo con la piel. No ingerir.

*Atención: El ácido okadaico es un producto tóxico, para su manejo es imprescindible el uso de guantes y trabajar con precaución.
Puede solicitar la hoja de seguridad del producto contactando con su distribuidor habitual o fabricante.

BIBLIOGRAFÍA

1.- 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.

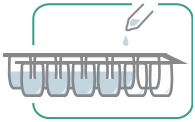
2.- .Smienk H., Calvo D., Razquin P., Domínguez E. & Mata L. *Single Laboratory Validation of A Ready-to-Use Phosphatase Inhibition Assay for Detection of Okadaic Acid Toxins. Toxins,* 2012, *5*, 339-352.

3.- 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*, 2013. 96, 1, 77-85.

OkaTest cumple con los requisitos del capítulo III A (4) a, b y c del Anexo III de la Regulación Europea (EC) 2074/2005 y puede ser usado como método complementario tal y como indica el Laboratorio de Referencia Europeo en su página web: http://aesan.msssi.gob.es/en/CRLMB/web/otros_procedimientos/other_crlmb_standard_operating_procedures.shtml

FLOWCHART PROCEDURE

ESQUEMA DEL PROCEDIMIENTO

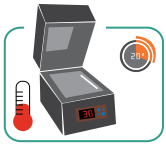


1. Add 50 µL samples/standars

1. Añadir 50 µL muestras/estándares

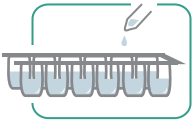
2. Add 70 µL Phosphatase Solution

2. Aplicar 70 µL Solución de Fosfatasa



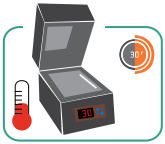
3. Incubate 20 min at 30ºC

3. Incubar 20 min a 30ºC



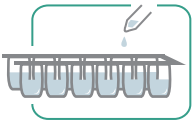
4. Add 90 µL Cromogenic Substrate

4. Añadir 90 µL Sustrato Cromogénico



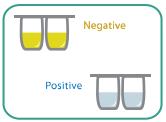
5. Incubate 30 min at 30ºC

5. Incubar 30 min a 30ºC



6. Add 70 µL Stop Solution

6. Añadir 70 µL Solución Stop



7. Read absorbance at 405 nm

7. Leer absorbancia a 405 nm

DSP PPIA kit-OkaTest

Single Laboratory Validation Report

1- EXECUTIVE SUMMARY

The **DSP PPIA (**commercial name **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 a rapid and simple method suitable for quantitative determination of the OA- toxins group from 63 to 352 µg of OA equivalents per Kg, including the maximum limit established as 160 µg of OA equivalents /Kg in the Commission Regulation of 29 April 2004 (Regulation (EC) 853/2004). Test applicable to shellfish species such as mussels, clams, oysters and scallops.

The **OkaTest** kit was developed by ZEULAB (previous name 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 fluorimetric detection.

A single laboratory validation was carried out at ZEULAB, followed by a collaborative study with 16 laboratories from 11 different countries. Both validations have been published in scientific journals; Toxins in 2012 by Smienk et al. and Journal of AOAC in 2013 by Smienk et al., respectively. Besides, OkaTest participates annually in intenational proficency exercises (Quasimeme, The Neatherlands).

OkaTest complies with the requirements established by the European Regulation (EC) 2074/2005 as complementary to the reference method.

http://www.aecosan.msssi.gob.es/en/CRLMB/web/public_documents/seccion/other_crlmb_standard_operating_procedures.htm.

Furthermore, **OkaTest** has been compared with other methodologies and using samples from the USA, UK and Argentina (Bich-Thuy et al., 2013, Turner & Goya, 2016 and Johnson et al., 2016).

This report shows the data obtained in the initial single laboratory validation (Smienk et al, 2012) that has been completed with additional information requested by the ISSC. Following a summary of the validation parameters:

Parameter	Results
Accuracy/Truness	98,00%
Measurement Uncertainty	14.92 - 31.08 µg equivalentes OA /kg
Precision	
Repeatibility:	1,4%- 3,9 % (Mean= 2,65%)
Reproducibility	0,8 %-17,7% (Mean= 6,45%)
Recovery	Okadaic acid: 78-114%
	DTX-1: 79-102%
	DTX-2: 83-94%
Working Range	63 - 352 µg equivalents OA /kg
Limit of Detection (LOD)	44 µg equivalets OA /kg
Limit of Quantification (LOQ)	56 µg equivalents OA/kg

2- METHOD PRINCIPLE AND SCOPE

DSP PPIA (OkaTest) is a protein phosphatase inhibition assay (PPIA), where the phophatase activity is ihnibited by the OA-toxins group, responsible for diarrheic shellfish poisoning (DSP). The PPIAs have been identified for a long time as an alternative for the detection of the OA-toxins, as Ser/Thr phosphatases are known to be their natural target (Bialojan & Takai, 1988). 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.

OkaTest is applicable to shellfish species such as mussels, clams, oysters and scallops. It is a quantitative method for determination of the OA- toxins group, where concentration of toxins present in the sample is calculated using a standard curve.

OkaTest includes five OA standards (0.5, 0.8, 1.2, 1.8 and 2.8 nM), phosphatase enzyme and substrate reagents ready to use.

The test procedure is extensively described in the user manual G-COM-OA.06.

3- VALIDATION

To evaluate the performance of the OkaTest kit, accuracy, uncertainty, precision, limit of detection and quantification were calculated. The assay temperature, incubation times and other variables affecting rugedness, together with specificity and matrix effects were also evaluated. Finally, a method comparison was carried out.

3.1 Accuracy/Trueness

To estimate the accuracy of the method 20 blank mussel samples (*Mytilus edulis*) were spiked with OA at 80, 120, 160, 240 and 300 µg/kg. Percentage of recoveries were calculated and are shown in Table 1.

Table 1. Recovery values from 20 different mussels samples spiked with OA at different levels along the working range. Mean (M), standard deviation (SD) and relative standard deviation (RSD). ND < 63 µg/kg

Theoretical Spike	µg OA equivalents/kg		Recovery	Mean	SD	RDS
	Before spiked	After spiked				
80	ND	73	91.3%			
80	ND	91	113.8%			
80	ND	87	108.8%	**112.5%**	**0.18**	**16.68%**
80	ND	112	140.0%			
80	ND	87	108.8%			
120	ND	133	110.8%	**106.7%**	**0.06**	**5.52%**
120	ND	123	102.5%			
160	ND	128	80.0%			
160	ND	169	105.6%	**98.8%**	**0.13**	**12.98%**
160	ND	173	108.1%			
160	ND	162	101.3%			
200	ND	186	93.0%			
200	ND	185	92.5%	**91.3%**	**0.30**	**0.27%**
200	ND	177	88.5			
240	ND	219	91.3%			
240	ND	205	85.4%	**96.1%**	**0.21**	**21.59%**
240	ND	195	81.3%			
240	66	304	126.7%			
300	ND	250	83.3%	**82.7%**	**0.01**	**1.14%**
300	ND	246	82.0%			

3.2. Measure of Uncertainty

Measurement of uncertainty was calculated using the results obtained in the accuracy experiment considering a confidence interval of 95%. Mean and standard deviation of the difference between the concentration of the spiked sample and the spiked amount were calculated. The coefficient of confidence (Z) and maximum error (E. max) were then determined (Table 2) according to the following equation:

$$E.\ max = Z_{\alpha/2} * SD/\sqrt{n},\ \text{where}$$

E. max: maximum error, **Z:** confidence coefficient; α 95% confidence interval**, SD**: standard deviation, **n**: number of samples.

Table 2. Estimation of uncertainty based on recovery data from 20 different mussels. ABS: absolute value of differences between OA concentration in spiked samples and spike concentration. Z= coefficient of confidence. SD= standard deviation. ABS E. max=absolute value of maximum error. ND < 63 µg/kg

Sample	Spike (OA µg/kg)	Blank Sample µg OA equiv. /kg	Spiked Sample	Recovery	ABS differences	Mean	SD	ABS E. Max
1	80	ND	73	91.3%	7	13	10.87	9.53
2	80	ND	91	113.8%	11			
3	80	ND	87	108.8%	7			
4	80	ND	112	140.0%	32			
5	80	ND	87	108.8%	7			
6	120	ND	133	110.8%	13	8	7.07	6.20
7	120	ND	123	102.5%	3			
8	160	ND	128	80.0%	32			
9	160	ND	169	105.6%	9			
10	160	ND	173	108.1%	13			
11	160	ND	162	101.3%	2	14	12.83	11.25
12	200	ND	186	93.0%	14			
13	200	ND	185	92.5%	15			
14	200	ND	177	88.5%	23	17	4.95	4.34
15	240	ND	219	91.3%	21			
16	240	ND	205	85.4%	35			
17	240	ND	195	81.3%	45			
18	240	66	304	126.7%	64	41	18.08	15.85
19	300	ND	250	83.3%	50			
20	300	ND	246	82.0%	54	52	2.83	2.48
				Mean	**23**			
				SD	**18.44**			
				ABS E. Max	**8.08**			

3.3. Precision

To determine the precision of the method, relative standards devidation (RSD) for repetibility and reproducibility were calculated.

To calculate repeatibility eight replicates of two mussel samples at two levels of concentration were analysed on the same day. Mean, standard deviation and relative standard deviation were calculated. The RSD obtained for the samples tested were, 1.4 and 3.9%, respectively. These values are far below the reference value of 15% (Horwitz W., 2002).

Table 3. Repeatability of 2 different mussel samples. Mean, standard deviation (SD) and relative standard deviation (RSD).

Repetition	Sample 1 (µg OA equiv./kg)	Sample 2 (µg OA equiv./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**
RSD	**1.4%**	**3.9%**

Intermediate precision/Reproducibility

Intermediate precision was estimated by testing 13 different samples (10 mussel samples and 3 from other species) at different levels of concentration on 3 different days by the same analyst (Table 4).

Mean values, standard deviation and relative standard deviation were calculated. An average of 6.45% of RSD was calculated for all the samples with different levels of concentration. Only sample 3, at a concentration below the regulatory limit showed a RSD above 15%, which is the variability expected for this concentration range (Horwitz, 2002).

Table 4. Reproducibility of thirteen different mussel (*Mytilus edulis*), king scallop (*Pecten maximus*) and clam (*Venerupis pullastra* and *V. vomboides*) samples. Mean, standard deviation (SD), relative standard deviation (RSD) were calculated.

Sample	Matrix	Day 1	Day 2	Day 3	Mean	SD	RSD
		µ OA equivalents /kg					
1	Mussel	82	94	90	**88**	6.17	**7.0%**
2	Mussel	106	95	90	**97**	8.05	**8.3%**
3	Mussel	98	101	72	**90**	15.95	**17.7%**
4	Mussel	109	106	95	**101**	7.80	**7.8%**
5	King Scallop	125	108	117	**117**	8.20	**7.0%**
6	Mussel	122	132	113	**122**	9.57	**7.8%**
7	Mussel	196	196	215	**202**	10.57	**5.2%**
8	Mussel	211	227	187	**208**	19.84	**9.5%**
9	Clam	261	251	260	**257**	5.51	**2.1%**
10	Mussel	257	250	258	**255**	4.36	**1.7%**
11	Mussel	250	253	281	**261**	16.90	**6.5%**
12	Mussel	277	279	289	**282**	6.62	**2.4%**
13	Clam	285	285	281	**284**	2.31	**0.8%**

The intermediate precision was also further evaluated in a collaborative study with 5 samples analysed by 16 different laboratories. Values of 11.2% and 13.2% were determined as the

highest relative standard deviation for repeatability and reproducibility, respectively (Smienk et al 2013).

3.4. Recovery

Recovery was calculated by spiking mussel and scallop samples (*Mytilus edulis* and *Pecten maximus*, respectively) with okadaic acid (OA) at 0.5, 1 and 1.5 times the regulatory limit. Samples were also spiked with 80, 160 and 240 µg/kg of DTX-1 and 80 and 160 µg/kg of DTX-2. Three to five repetitions of each concentration were analysed on different days. Results are shown in Tables 5 and 6.

Table 5. Results (µg OA equivalents/kg) from recovery of OA in mussel and scallop samples at 80, 160 and 240 µg/kg. Standard deviation (SD), relative standard deviation (RSD) and recovery were calculated. ND= <63 µg/kg).

	Mussel				King Scallop			
	spiked OA (µg/kg)							
Repetition	**0**	**80**	**160**	**240**	**0**	**80**	**160**	**240**
1	86	158	230	271	ND	82	162	252
2	87	134	211	282	ND	84	142	218
3	87	178	216	257	ND	89	150	268
4	95	193	253	298	ND	102	177	268
5	95	191	257	280	ND	99	158	271
Mean	**90**	**171**	**233**	**277**	**-**	**91**	**157**	**255**
SD	4.8	25.0	20.9	15.1	-	9.0	13.3	22.2
RSD	5.4%	14.6%	8.9%	5.4%	-	9.9%	8.4%	8.7%
Recovery	**-**	**101%**	**90%**	**78%**	**-**	**114%**	**98%**	**106%**

Table 6. Results (µg OA equivalents/kg) from recovery of DTX-1 and DTX-2 in mussel and scallop samples spiked at 80, 160 and 240 µg/kg. Mean, Standard deviation (SD), relative standard deviation (RSD) and recovery were calculated. ND= <63 µg/kg).

	King scallop				Mussel					
	spiked DTX - 1 (µg/kg)				spiked DTX2 (µg/kg)					
Repetition	**0**	**80**	**160**	**240**	**0**	**160**	**0**	**80**	**0**	**160**
1	ND	63	101	211	ND	145	86	157	ND	128
2	ND	91	127	179	ND	156	101	163	ND	130
3	ND	81	132	175	ND	151	-	-	ND	124
4	ND	82	132	261	-	-	-	-	-	-
5	ND	93	140	228	-	-	-	-	-	-
Mean	**ND**	**82**	**126**	**211**	**ND**	**151**	**93.5**	**160**	**ND**	**127**
SD	-	11.9	14.8	35.6	-	5.5	-	4.2	-	2.7
RSDr	-	14.5%	11.7%	16.9%	-	3.7%	-	2.7%	-	2.1%
Recovery	**-**	**102%**	**79%**	**88%**	**-**	**94%**	**-**	**83%**	**-**	**80%**

The mean of recoveries obtained for the different concentrations tested and toxins were acceptable and ranged from 78 to 114%.

3.5. Specificity

Specificity was studied by determining the possible interferences caused by other lipophilic toxins such as Azaspirazides (AZA), Yessotoxins (YTX) and Pectenotoxins (PTX).

A mussel sample naturally contaminated was spiked, on two different days, with 160 µg/kg of AZA-1 (NRC, Institute for Marine Biosciences, Canada), 160 µg/kg PTX-2 (Cifga laboratories, Spain) and 1000 µg/kg of YTX (NRC, Institute for Marine Biosciences, Canada) and concentration of OA determined following the kits´ instructions. Results obtained for spiked and non-spiked samples were very similar and within the method variability, showing no interferences by the toxins tested.

Table 6. Results obtained from spiking a mussel sample with 160 µg/kg of azaspirazides (AZA), 160 µg/kg of pectenotoxins (PTX) and 1000 µg/kg of yessotoxins (YTX).

Spiked Mussel	Day 1	Day 2
	µg equiv. OA /kg	
0	82	82
160 (µg/kg) **PTX-2**	83	79
160 (µg/kg) **AZA-1**	82	73
1000 (µg/kg) **YTX**	82	82

3.6. Working Range and Linear Ranges

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.

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).

The linearity of an assay was 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 curves used to calculate the concentration of these standards from their absorbances (Table 7).

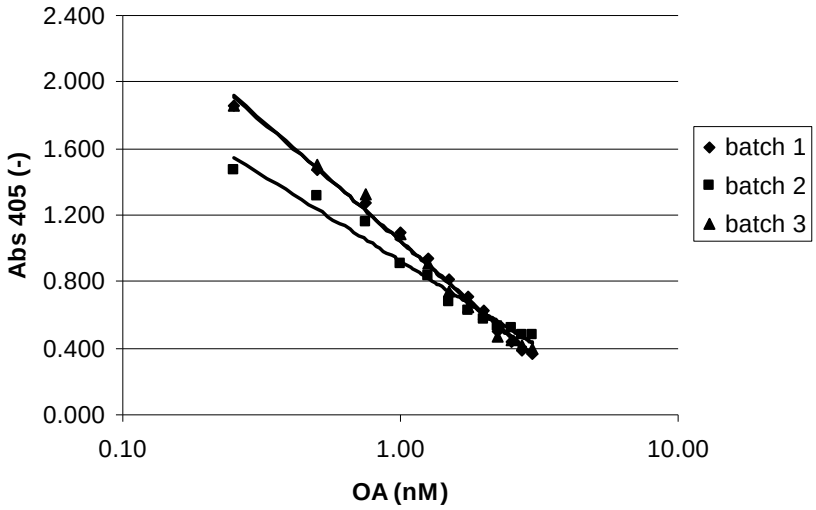


Fig 1. Working range of the assay for 3 different phosphatase batches. R^2: 0.99, 0.98 and 0.99 for batch 1, batch 2 and batch 3, respectively. Working range from 0.25 to 3.0 nM OA.

Table 7. 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.

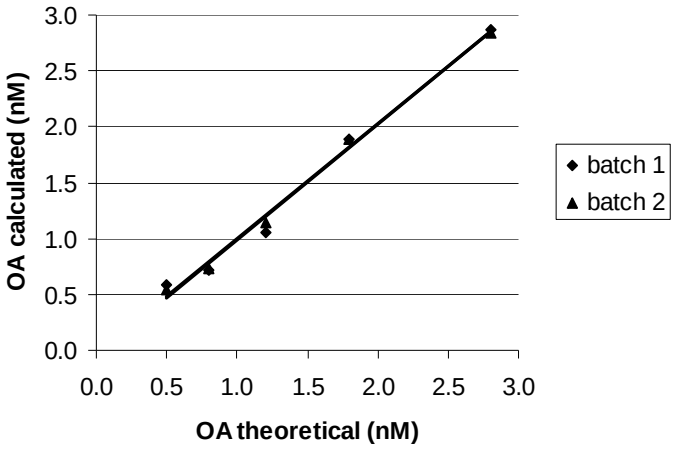


Fig 2. Comparison of the theoretical and calculated standard concentration. Concentration were `backcalculated´ by using the standard curve obtained with batch 1. R^2: 0.99 and 1.00 for batches 1 and 2, respectively.

The linearity was also determined by testing 10 blank mussel samples spiked at 80, 160, 200, 240 and 300 µg/kg (Table 8). OA concentrations obtained were divided by the spiked concentration (relative recovery). Mean of relative recovery per concentration was plot against the spiked concentration and curve equation to observe the relative response (Figure 3).

Table 8. Assay linerarity. Results obtained from 10 blank samples spiked with 80, 120, 160, 200 and 240 µg/kg to determine linearity of the assay.

Spike OA µg/kg	After spiked µg OA equiv. /kg	Relative recovery	Mean Relative Recovery
80	91	1,14	1,11
80	87	1,09	
160	169	1,06	0,96
160	162	1,01	
200	186	0,93	0,91
200	177	0,88	
240	219	0,91	0,88
240	205	0,85	
300	250	0,83	0,83
300	246	0,82	

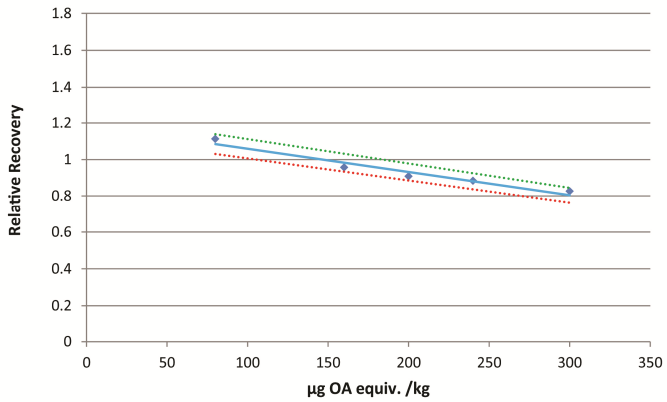


Figure 3. Assay linearity. Relative recovery data plot against spiked OA concentration µg/kg in solid blue line. Green and red dotted lines were obtained by multiplying the OA concentration by 0.95 and 1.05.

3.7. Limit of detection and Limit of quantification

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).

Table 9. Quantification of the standard solvent (10 repetitions) as OA concentration equivalents (µg/kg) to estimate the LOD and LOQ. Mean, standard deviation (SD) and relative standard deviation (RSD).

Repetition	µg OA equivalents /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
RSD	4.6%
LOD	**44**
LOQ	**56**

3.8. Ruggedness

The influence of different experimental conditions critical for the kits' performance such as assay temperature, incubation times or reaction component volumes were evaluated. The ruggedness between batches with spiked mussel samples was also evaluated.

3.8.1- 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 10).

Table 10. Influence of the assay temperature on the results of the test. The mean, standard deviation (SD) and relative standard deviation (RSD) were calculated.

Sample	28 ºC	30 ºC	32 ºC	mean	SD	RSD
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 RSD were below the 15%, variation that can be expected at this concentration (Horwitz, 2002).

3.8.2- 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 11).

Table 11. Influence of time on the first incubation of the assay. The mean, standard deviation (SD) and relative standard deviation (RSD) were calculated.

Sample	- 2 min	0	+ 2 min	+ 4 min	mean	SD	RSD
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 RSD was 2.9% at highest, a bit lower than the ones obtained for the first incubation time (Table 11).

Table 12. Influence of the incubation time (2nd incubation with the substrate) on the assay. The mean, standard deviation (SD) and relative standard deviation (RSD) were calculated.

Sample	- 3 min	0	+ 3 min	+ 6 min	mean	SD	RSD
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%

3.8.3- 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 RSD and error were evaluated (Table 13).

Table 13. Effect of the sistematic pipetting error on the results of the test. Mean, standard deviation (SD), relative standard deviation (RSD) and error (Errormax) were calculated.

Sample	- 2 µL	0	+ 2 µL	mean	SD	RSD	E. Max*
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%)

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

The RSD 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 14).

Table 14. Effect of a single pipetting error on the results of the test. Mean, standard deviation (SD), relative standard deviation (RSD) and error (Errormax) were calculated.

Variable	-5 µL	0	+ 5 µL	mean	SD	RSD	E Max*
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%)

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

Table 14 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 14 also shows that high RSDr values (above 10%, ZEULAB in-house 5%) are a good indication for pipetting error. Substrate and stop solution pipetting errors seem to be much less important RSDr < 10%.

3.8.4- 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 RSD was evaluated (table 15).

Table 15. 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 (RSD) were calculated.

Sample	30 min	60 min	90 min	Mean	SD	RSD
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 RSD values obtained were at highest 5.0% and comparable to those obtained for within batch variability (see table 15).

3.8.5- Ruggedness between batches in samples

The ruggedness of the assay with molluscs samples was also determined. Ten blank mussel samples were spiked at 80, 120, 160, 200 and 240 µg/kg of okadaic acid and tested following the kits´ instructions in two different days and using two different batches. Differences between concentrations obtained in each batch for the different samples were calculated. Mean and standard deviation of the differences together with the experimental t-score and critical t values were also determined (Table 16).

$$t\,exp = \frac{|Mean|}{\frac{s}{\sqrt{n}}}$$

Mean ≡ mean of the difference of skewness

s ≡ Standard deviation; n ≡ number of samples

The critical value was calculated for a significance of α = 0.05 (95% confidence) for n-1 degrees of freedom. If the calculated value of experimental-t is less than the critical-t, we can affirm that the hypothesis is true, so that there is an equivalence between both methods.

The experimental t-score was smaller than the critical t-value (t exp< t crit; 1.42<2.26); and so the range of skewness was acceptable. There is not significant difference between batch 1 samples and batch 2 concentrations.

Table 16. Results from testing 10 different mussel samples spiked at different concentrations and tested with two different batches in two different days. Mean, standard deviation (SD), experimental-t and critical-t values were calculated.

Sample	Batch No. 1	Batch No. 2	Differences between batches
	OA equivalents µg/kg		
1	91	74	-17
2	87	79	-8
3	133	102	-31
4	123	150	27
5	169	145	-24
6	162	177	15
7	186	177	-9
8	185	168	-17
9	219	174	-45
10	159	169	10
		Mean	**-9.9**
		SD	**22.01**
		Experimental t-score	**1.42**
		Critical t-value	**2.26**

The data was also analyzed using a Welch's test or unequal variances t-test, which is a two-sample location test used to check the hypothesis that two populations have equal means (H_0).

Therefore, considering that the null hypothesis (H_0) refers to the fact that the two batches do not show differences in the analysis of samples spiked with a known concentration of okadaic acid. Mean, variance and p-value were calculated (Table 17).

P-value (0.603) was higher than 0.05 (0.603>0.05), therefore we do not reject the null hypothesis. The observed difference between the sample´s means is not convincing enough to say that the average value between both batches differing significantly.

Table 17. Mean, variance and p-value calculated for results obtained from 10 spiked mussel samples tested with bath 1 and batch 2 of OkaTest (results from Table 16).

	Batch 1	Batch 2
Mean	151.40	141.50
Variance	1812.93	1682.50
p value	0.603	

Residual values analysis evaluates the goodness of the test. A linear relationship is confirmed when the residues have symmetry around zero and a homogeneous random dispersion. Graphical representation is the most common methodology, being a very visual and simple method to evaluate symmetry. Residual standard values were also calculated (table 18) and

the distribution plotted. The adjustment is adequate since the residual values have a random and homogeneous distribution around 0, being between ± 2 (Figure 4).

Table 18. Residual standard values obtained for OA concentration results obtained for 10 mussel samples analysed with two different batches of OkaTest.

Sample	Residual standard values
1	5.51E-05
2	-0.39
3	0.79
4	-1.67
5	0.67
6	-0.98
7	0.13
8	0.46
9	1.78
10	-0.79

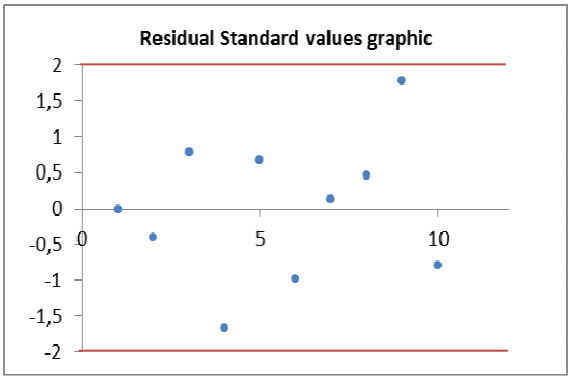


Figure 4. Distribution of residual standard values obtained for OA concentration results obtained for 10 mussel samples analysed with two different batches of OkaTest

3.9- Matrix Effects:

To determine the matrix effect 10 different molluscs' samples were tested according to the kit instructions and further diluted; where the final concentration of diluted samples was calculated multiplying by the appropriate dilution factor. Mean and SD of the differences between concentrations for diluted samples were calculated.

To evaluate if the concentrations obtained for diluted samples were within the assay variability and not due to matrix effect the experimental t-score and t-critical values were calculated (Table 19):

As the experimental t-score is smaller than the critical t-value (0.93<2.26) the skewness obtained is acceptable and does not indicate matrix effect.

Table 19. OA equivalents µg/kg for 10 mussel samples tested a two different dilutions. Mean, standard deviation (SD), experimental-t and critical-t values were calculated.

Sample	Dilution 1	Dilution 2	Diferences
	OA equiv. µg/kg		
1	40	37	-3
2	980	974	-6
3	29	30	1
4	620	628	8
5	595	560	-35
6	138	104	-34
7	1192	1287	95
8	1149	1318	169
9	118	108	-10
10	85	87	2
		mean	**18.7**
		SD	**63.84**
		Experimental- t score	**0.93**
		Critical-t value	**2.26**

3.10. Method comparison

A method comparison was performed with the mouse bioassay (MBA), reference method in Europe until 2011 and LC-MS/MS (current official reference method in Europe).

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

Twenty-three out of thirty-one samples tested positive for both methods and five samples were negative for both methods. However, three samples were positive for MBA and negative for OkaTest (Table 20). 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 also tested by LC-MS/MS where two out of three results were above the regulatory limit as well by MBA, showing slightly higher quantifications compared to OkaTest (185, 152 and 177 OA toxins µg/kg, respectively).

OkaTest was compared with LC-MS/MS for a total of 69 samples, where results from the reference method came from two different laboratories (Tables 20 and 21).

Table 20. Results from MBA, OkaTest and LC-MS/MS. Positive results (+): ≥160 µk/kg. Negative result (-): <160 µg/kg. *HPLC-MS results were not with toxicity factors. However only 4 samples contained DTX-2. LC-MS/MS carried out at the Reference Laboratory in Vigo.

Sample	Matrix	MBA	OkaTest	OkaTest µg OA equiv. /kg	LC-MS/MS µg OA /kg
1	**Mussel**	-	-	122	ND
2	**Scallop**	-	-	ND	ND
3	**Mussel**	-	-	ND	ND
4	**Donax**	-	-	97	82
5	**Cockle**	-	-	ND	ND
6	**Mussel**	+	+	196	158
7	**Mussel**	+	+	232	502
8	**Mussel**	+	+	268	ND
9	**Scallop**	+	+	264	184
10	**Mussel**	+	+	250	177
11	**Mussel**	+	+	265	288
12	**Mussel**	+	+	196	318
13	**Mussel**	+	+	>377	604
14	**Mussel**	+	+	>377	894
15	**Mussel**	+	+	277	390
16	**Mussel**	+	+	305	658
17	**Mussel**	+	+	306	414
18	**Mussel**	+	+	310	392
19	**Mussel**	+	+	>377	444
20	**Mussel**	+	+	315	329
21	**Mussel**	+	+	270	232
22	**Mussel**	+	+	277	235
23	**Mussel**	+	-	135	152
24	**Mussel**	+	+	164	98
25	**Mussel**	+	+	211	168
26	**Mussel**	+	+	251	209
27	**Mussel**	+	+	191	113
28	**Mussel**	+	-	124	177
29	**Cockle**	+	+	252	193
30	**Mussel**	+	+	216	247
31	**Mussel**	+	-	144	185
32	**Mussel**		-	ND	ND
33	**Mussel**		+	>377	357
34	**Mussel**		-	ND	292
35	**Mussel**		-	ND	ND
36	**Mussel**		-	ND	ND
37	**Mussel**		+	304	316

A comparison of OkaTest and the reference method LC-MS/MS was made for those samples which showed a quantitative value with both methods. The samples were analyzed by paired t-test to determine the equivalence of the two analytical methods, comparing both means to determine if the difference between the expected means surpasses the one produced randomly.

The hypothetical difference of Means should be zero (Null hypothesis H_0), which means that both methods are considered equivalents.

Table 21. Analysis t Student match pairs from results OkaTest and LC-MS/MS results from table 20.

	OkaTest	LC-MS
Mean	240.33	281.71
t-statistic	1.74	
P(T≤t) value (probability value) for the t-statistic (one-tailed)	0.048	
Critical value of a t-distribution (one-tailed)	1.72	
P(T≤t) value (probability value) for the t-statistic (two-tailed)	0.097	
Critical value of a t-distribution (two-tailed)	2.09	

The null hypothesis was accepted because critical- t two-tail < t Stat < t Critical two-tail (-2.09 < -1.74 < 2.09) and p (0.097)>0.05. The observed difference between the sample means (240.33 and 281.71) was not convincing enough to say that the average value between LC-MS and Okatest differ significantly.

Besides, the test t was applied manually to the difference of values obtained for each sample. For this application, the value of the experimental t-score statistic was calculated, as well as the critical t- value:

We could affirm that the hypothesis is true because the calculated experimental-t value was smaller than the critical-t value (1.65<2.08). The skewness is acceptable and the methods Okatest and LC-MS/MS are considered to be similar (Table 22).

Table 22. Mean, standard deviation (SD), experimental-t and critical-t values were calculated.

Mean	37.77
SD	107.56
Number of samples	22
Experimental t-score	1.65
Critical-t value	2.08

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

Mussels' results were statistically analyzed by applying a t-Student match pairs test to the results above the limit of quantification for each method.

The null hypothesis was accepted because the critical-t two-tail < t Stat < Critical-t two-tail (-2.37 < 0.94 < 2.37) and p (0.8) > 0.05. Therefore, we do not reject the null hypothesis. The observed difference between the sample means (167.75 and 165.00) is not convincing enough to say that the average value between LC-MS and Okatest differ significantly (Table 24).

Table 24. Analysis t Student match pairs from results OkaTest and LC-MS/MS results from table 22:

	OkaTest	LC-MS
Mean	167.75	165.00
t-statistic	0.94	
P(T≤t) value (probability value) for thet-statistic (one-tailed)	0.19	
Critical value of a t-distribution (one-tailed)	1.89	
P(T≤t) value (probability value) for the t-statistic (two-tailed)	0.38	
Critical value of a t-distribution (two-tailed)	2.36	

We applied the test t manually to the difference of values obtained for each sample. For this application the value of the experimental t-score statistic was calculated, as well as the critical-t value (Table 25).

Table 25. Mean, standard deviation (SD), experimental-t and critical-t values were calculated.

Mean	-2.75
SD	8.26
Number of samples	8
Experimental t-score	0.94
Critical-t value	2.36

We could affirm that the hypothesis is true because the calculated value of experimental-t is smaller than the critical-t (0.94<2.36). The skewness is acceptable and the values obtaines by Okatest and LC-MS/MS are considered similars (Table 23).

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Article

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

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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.

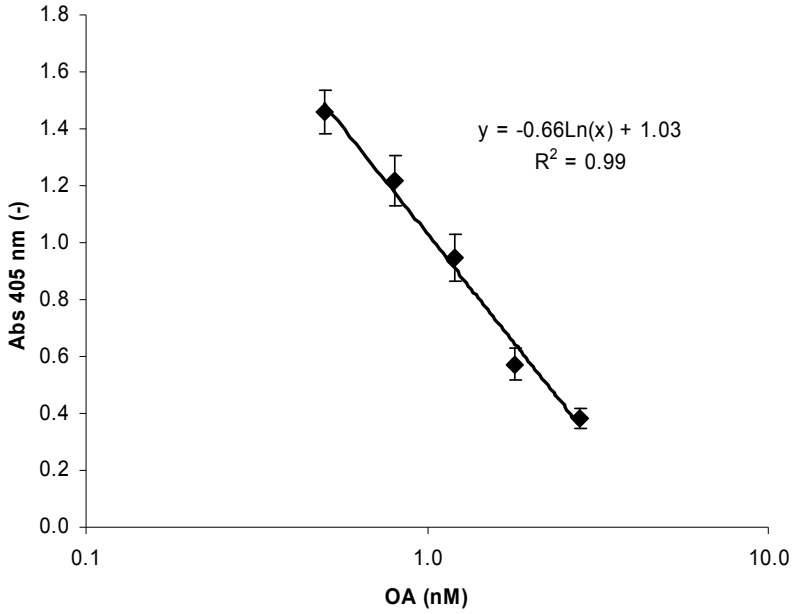
As MBA is a qualitative method, results obtained by OkaTest and LC-MS/MS were interpreted qualitatively for comparison purposes. Therefore, samples with a concentration ≥160 µg/kg were regarded as positive, while samples with a concentration <160 µg/kg were reported negative.

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.

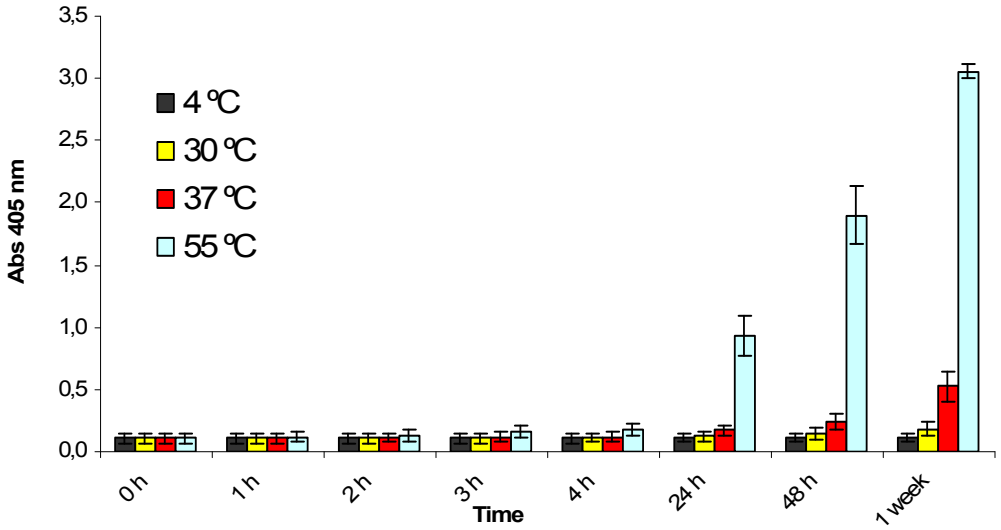
OA theoretical (nM)	OA calculated (nM)	Relative Absolute Difference
0.50	0.55	9.0%
0.80	0.83	3.8%
1.20	1.21	0.8%
1.80	1.78	1.1%
2.80	2.73	2.5%

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.

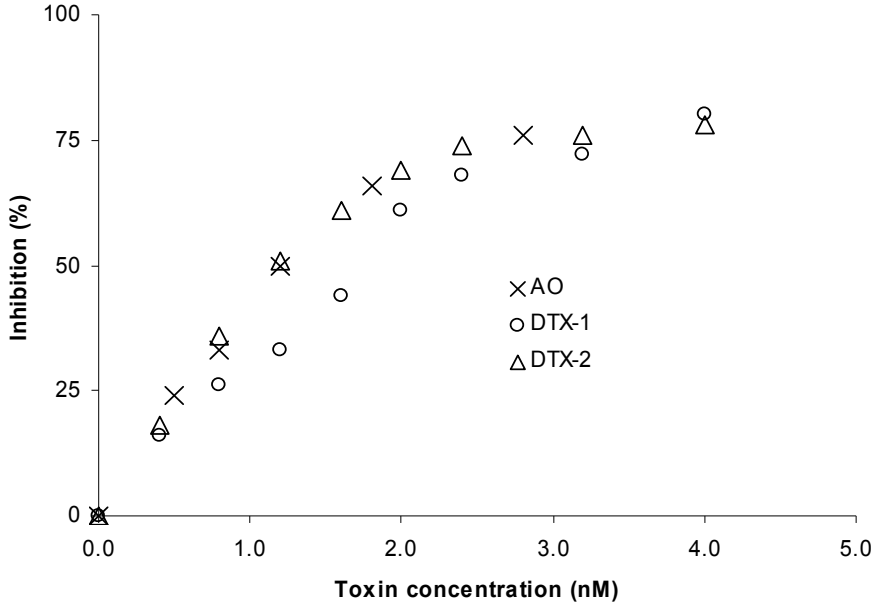


Table 6. Recovery of the different toxins was calculated testing 5 samples at 0.5, 1 and 1.5 times the regulatory limit on 3 different days. OA Certified Reference Material (NRC CRM-OA-c) was spiked on mussel and king scallop. DTX-1 and DTX-2 were spiked on king scallop. ND: not determined.

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.

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

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FOOD CHEMICAL CONTAMINANTS

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

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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

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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).

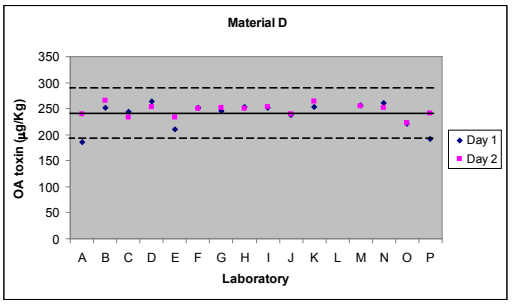
Samples with an OA concentration falling outside the working range (<0.5 nM or >2.8 nM) will be reported as <63 µg/kg (or <0.5 nM) or >352 µg/kg (or >2.8 nM), respectively.

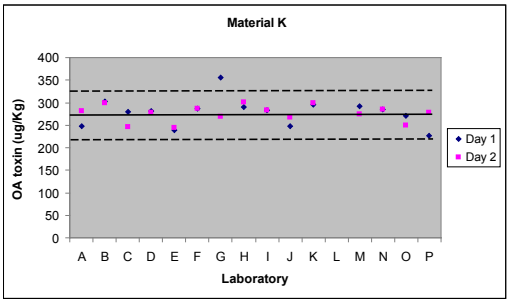
Results were recorded by each participant in a tailor-made Excel spreadsheet with which the results were automatically calculated when the absorbance values were entered. All participants sent back an electronic copy of the reporting sheet for each day of analysis.

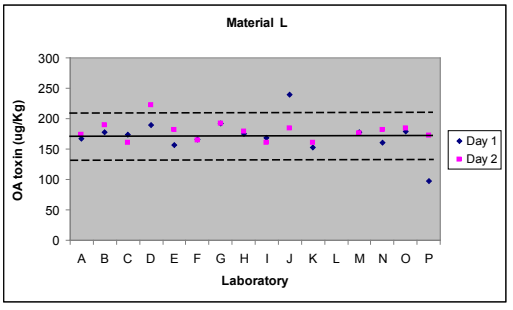
Table 6. Individual results (µg OA total equivalents/kg) reported from laboratories A to P for Materials A, D, E, F, G, K, L, and N on Days 1 and 2. Invalid or incorrect results are those in bold type.

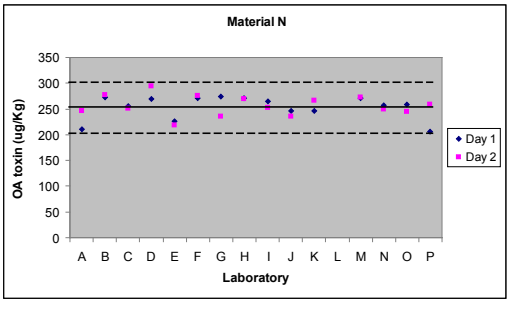
	µg OA total equivalents/kg															
	Material															
	A		D		E		F		G		K		L		N	
	Day															
Lab	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
A	<63	<63	186	239	97	102	<63	<63	<63	<63	248	281	167	174	210	247
B	<63	<63	251	266	100	101	<63	<63	<63	<63	302	299	177	190	273	277
C	<63	<63	244	233	96	87	<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.









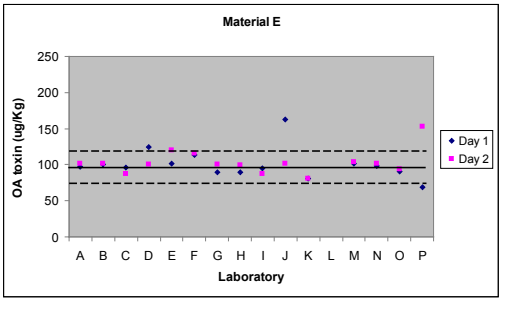


Figure 1. Individual results for each test material obtained per lab and per day of analysis (including outliers). The solid line shows the assigned mean value calculated in this study for each material. The dashed lines indicate the theoretical reproducibility SD determined for each material in this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

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