METHOD FOR DETECTION OF OKADAIC ACID-TOXINS GROUP IN EDIBLE MOLLUSCS

STANDARD OPERATIONAL PROCEDURE

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07/05/10	1	1	Document identification	H Smienk
07/05/10	1	2	Table of summary of changes	H Smienk
07/05/10	1	11	References included	H Smienk
07/05/10	1	12,13	Appendix I and II added.	H Smienk
14.09.10	2	7	Advice the use or blender or ultraturrax if sample is not fully homogenized after vortex	H Smienk
14.09.10	2	9	Specify that adhesive films should be removed	H Smienk
14.09.10	2	9, 13	Include more details for pipette calibration and ask to record results in Reporting Sheet	H Smienk
13.10.10	3	4, 8	Remove stop solution from kit contents and procedure	H Smienk
15/10/10	3	7	Use of ultra homogenizer during sample prep	H Smienk
16/10/10	3	3, 5, 7, 8	Minor changes	H Smienk
16/10/10	3	4	Send 1 vial of spiking solution	H Smienk
02/05/11	4	all	Change of kit name before Toxiline-DSP.Co, now Okatest	H. Smienk

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1. INTRODUCTION

As stated in the Regulation (EC) Nº 853/2004, marine biotoxins such as okadaic acid (OA) and dinophysistoxins (DTXs) pose a serious hazard to human health when present above certain limits in shellfish. These toxins, known as OA-toxins group, are the main cause of Diarrhoeic Shellfish Poisoning (DSP), a human gastrointestinal disease caused by the ingestion of contaminated shellfish. The OA-toxins groups are also described to be involved in tumour promotion.

Commission Regulation (EC) No 2074/2005 specifies the recognised testing methods for marine biotoxins. In the case of lipophilic toxins and, among them, OA-toxins, it is indicated that bioassays, using mice or rats, are the reference methods for detecting and preventing toxic shellfish from being harvested. There is however a clear interest for its replacement by alternative methods because of its low sensitivity, variability of response, presence of false positives and ethical problems. It also states that the use of a series of methods, such as high-performance liquid chromatography (HPLC) with fluorimetric detection, liquid chromatography with mass spectrometry (LC-MS), immunoassays and functional assays, such as the phosphatase inhibition assay, shall be used as alternatives or supplementary to the biological testing methods, provided that either alone or combined they are not less effective than the biological methods and that their implementation provides an equivalent level of public health protection. Therefore, alternative methods should be accepted if their performance has been validated according to an international protocol.

2. SCOPE

This protocol specifies a method for the quantitative determination of OA and other lipophilic toxins of the OA group including DTX1, DTX2, and their ester forms by a colorimetric phosphatase inhibition assay. This method is applicable to shellfish species such as mussels, clams, cockles and scallops.

3. PRINCIPLE

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

4. KIT CONTENTS

4.1 96 wells transparent microtiter plate (12 strips of 8 wells), two adhesive foil sheets

4.2 4 Vials of Phosphatase

4.3 5 Vials with Okadaic Acid Standards (0.5, 0.8, 1.2, 1.8 and 2.8 nM)

4.4 1 Chromogenic substrate

4.5 1 Phosphatase Dilution Buffer

4.6 1 Stock Buffer Solution

5. REAGENTS TO BE SEND WITH THE KIT

5.1 OA Spiking solution 1 vial

5.2 Sample labelled “familiarization sample”

6. REAGENTS

6.1 Methanol (Reagent grade)

6.2 HCl (Reagent grade, 37 % v/v)

6.3 NaOH (Reagent grade)

6.4 Deionised water (type II, ISO 3696)

7. EQUIPMENT

7.1 Micropipettes (adjustable 100 µL or 200 µL and adjustable 1000 µL pipette)

7.2 Ultra homogenizer

7.3 Block heater or incubator for 30 ºC ± 2 ºC

7.4 Microwell absorbance reader (405 nm ± 10 nm wavelength filter).

7.5 Water bath at 76 ºC ± 2 ºC

7.6 Graded 50 mL centrifuge tubes

7.7 Laboratory glassware: 25 and 250 mL graduated cylinder, 1000 mL graduated cylinder, 1000 mL volumetric flask, 25 mL glass pipettes

8. SOLUTIONS

Note: The material and equipment indicated can be replaced for material/equipment with equal or higher precision. If different volumes are required for any of the solutions mix the reagents using the same rate.

8.1 Extraction solvent: Methanol (100% v/v)

8.2 1 X Buffer solution: Mix 20 mL of the **stock buffer solution** (see paragraph 4.6) with 180 mL of deionised water (see paragraph 6.4) using 25 mL and 250 mL graduated cylinder, respectively. Keep at 4 ºC - 12 ºC while running the assay and not in use. This is enough for 9 samples.

8.3 2.5 M HCl: Take 205 mL of HCl (37 % v/v) (see paragraph 6.2) using a volumetric cylinder of 500 mL and add with caution to 400 mL of distilled water already contained in a volumetric flask. Mix and add deionised water for a final volume of 1000 mL.

8.4 2.5 M NaOH: Add 100 g of NaOH (see paragraph 6.3) to 500 mL water and dissolve. Transfer to a **volumetric flask** and add deionised water for a final volume of 1000 mL.

8.5 Phophatase Solution

This solution must be used immediately after preparation. Add 2.0 mL of the **phosphatase dilution buffer** to each of the phosphatase vials needed. To make sure that the enzyme is fully hydrated mix gently during 1 h ± 5 min. at room temperature (22 ºC ± 2 ºC) on a roller mixer or a shaker at 60 rpm. maximum. **Do not use the vortex at any moment**. Each enzyme vial contains enough volume for 24 wells. If more than one vial is required, dissolve each vial as described above, make a pool with the content of these vials and mix gently by inversion before use.

8.6 Okadaic Acid Standards (0.5, 0.8, 1.2, 1.8 and 2.8 nM)

Note: In order to correctly quantify the amount of okadaic acid in the samples it is important to pipette accurately. Make sure that the pipettes are checked for accuracy before use. The okadaic acid standards are dissolved in an aqueous buffer. It is really important to mix well by vortexing before applying to make sure these solutions are homogeneous.

8.7 Chromogenic Substrate

This is a ready-to-use substrate. In order to maintain its working conditions do not pipette directly from the bottle but first dispense the volume you need in transparent labware (for example glass 5 mL test tubes). The substrate contains a stabilization resin. Make sure not to add this resin to the microwells. Do not use this solution if the absorbance at 405 nm of 90 µL of this solution in a microwell rises above 0.6.

9. STABILITY AND STORAGE

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

10. SAFETY

Safety clothing should be worn and skin contact with the reagents avoided. Do not ingest.

A SAFETY DATA SHEET is available from ZEU-INMUNOTEC on request.

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

11. SAMPLE PREPARATION

Warning: *It is recommended to maintain the laboratory temperatures between 20ºC and 24 ºC during the extraction process. Use tubes with screw caps for extraction and hydrolysis and keep the tubes closed at all times to avoid methanol evaporation.* ***If not in use, store the samples frozen****.*

11.1 Thaw the coded test samples (5.0 ± 0.1 g of homogenate mollusc) at room temperature (22 ºC ± 2ºC) for a maximum of 30 minutes ± 5 minutes and add 25 mL of **extraction solvent** (see paragraph 8.1) with a 25 mL graduated cylinder to each of the 50 mL tubes. Mix for 2 minutes using an Ultraturrax **and check visually for good homogenization.**

11.2 Centrifuge at 2000 g for 10 min at 4 ºC. The supernatant is called “methanolic extract”.

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

11.3 Take 640 µL of the methanolic extract, put it in a 50 mL graded centrifuge tube and add 100 µL of **2.5 M NaOH** (see paragraph 8.4).

11.4 Seal the test tube and heat at 76 ± 2 ºC for 40 minutes in a water bath.

11.5 Do not cool down the sample, but add 80 µL of **2.5 M HCl** (see paragraph 8.3) immediately after.

11.6 Add 19.18 mL of **1 X buffer solution** (see paragraph 8.2) with a glass pipette up to a total volume of 20 mL

12. ASSAY PROCEDURE

Warning:

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

1. Pippettes should be calibrated before the assay (follow Appendix I for calibration instructions and record the results on the Reporting Sheet provided-OKATEST-VALIDATION-REPORTING SHEET.xls).
2. Use pipettes according to the volumes to be dispensed. Use pipettes with a maximum pipette volume of 100 µL or 200 µL for volumes below 200 µL.
3. It is important to organize well the working space to be able to respect the different assay times.
4. Be sure that the incubator's temperature is stabilized before its usage.

12.1 Samples and standards must be analysed in **duplicate**. For this purpose add 50 µL of each sample (see paragraph 11) and standard (see paragraph 4.3) to two wells.

12.2 Immediately after adding the samples and/or standards, add 70 µL of phosphatase solution (see paragraph 8.5) to each well. **The phosphatase addition should not take longer than 5 minutes.** Cover the plate with the adhesive film provided and mix by gentle tapping on the side. **Do not use a plate shaker at any time.**

12.3 Incubate at **30 ºC ± 2 ºC** for 20 minutes ± 0.5 minutes .

12.4 Remove the adhesive film, and add **90** µL of **Chromogenic Substrate** (see paragraph 8.7) to each well and mix by tapping gently on the side. **Substrate addition should not take longer than 5 minutes**. Cover the plate with the adhesive film. **Do not use a plate shaker at any time**.

12.5 Incubate at **30 ºC ± 2 ºC** for 30 minutes ± 0.5 minutes.

12.6 Read absorbance of samples and standards:

Absorbance wavelength: 405 nm ± 10nm

13. GRAPHIC REPRESENTATION AND CALCULATION OF RESULTS

13.1 Obtain a standard curve:

Plot the absorbance on a linear *y axis* and the concentration of okadaic acid in a logarithmic x *axis* and use a logarithmic fitting as shown below:

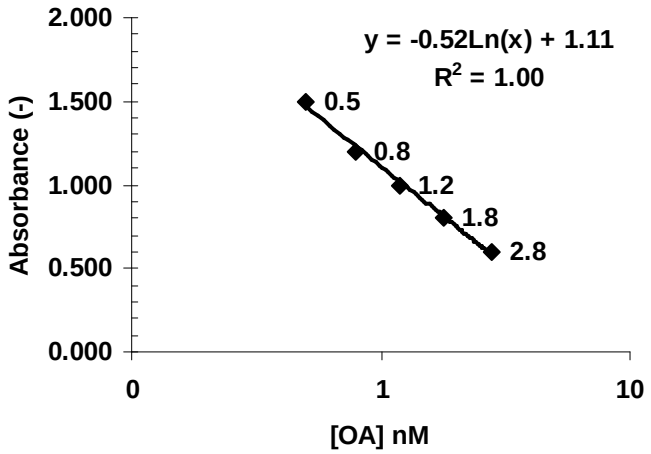

Fig 1: Logarithmic fitting of the standard curve

The R^2 has to be higher than or equal to **0.96**.

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

$$y = a\ ln(x) + b$$

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

For example:

$$x = e^{\frac{(y-b)}{a}}$$

13.3 Calculate the OA-toxin group concentration in tissue as follows:

$$\textbf{Ct (µg/kg)} = \frac{\textbf{\textit{Cs (nM) x FD x MW (g/mol) x Ve (l)}}}{\textbf{\textit{Mt (g)}}}$$

Ct: OA-toxin group concentration in tissue; **Cs**: OA-toxin group toxin concentration in sample**; FD**: Methanolic extract dilution factor (i.e. 20/0.64 → x31.25); **MW**: Okadaic acid molecular weight = 805; **Ve**: Methanolic extract volume (0.025 L); **Mt:** Tissue weight (5g); Example: (1.5 nM x 31.25 x 805g/mol x 0.025L)/5g = 189 ug/kg.

For samples with a toxin concentration outside the range of the calibration curve (<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).

- **In order to make easier the calculation an Excel sheet (**OKATEST –Reporting Sheet.xls) **will be provided**.

14. REFERENCES

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2. COMMISSION REGULATION (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. *Official Journal of the European Communities*, L338: 27-59.
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6. Takai A and Mieskes. Inhibitory effect of okadaic acid on the p-nitrophenyl phosphate phosphatase activity of protein phosphatases., *Biochem. J*. 275, pp 233-239, 1991.
7. Tubaro A, Florio C, Luxich E, Sosa S, Della Loggia R, Yasumoto T. A protein phosphatase 2A inhibition assay for a fast and sensitive assessment of okadaic acid contamination in mussels. Toxicon. 34(7):743-52, 1996.

APPENDIX I

Verification of pipette calibration

The correct calibration of the automatic pipettes can be verified by weight using liquid distilled/deionised and degasified water as a reference.

Reagents and materials

1. Pipette tips
2. Beakers
3. High precision balance (4 significant figures)
4. Deionised and degasified water

For degasifying the water can be autoclaved. Care should be taken, however, that the material used, including the water, is at room temperature. The density of the water at 20 ºC = 0.9982.

The most critical volumes of the OkaTest test are the small volumes used in the preparation (e.g. 50 µL) and the test itself (e.g. 50, 70 and 80 µL). Calibration of the pipette at 50 and 100 µL is sufficient, although for normal calibration procedures the pipettes are verified at the highest and lowest volume that the pipette is capable of pipetting. For verification of the pipettes the volumes should be weighted 5 times and the mean values, errors and coefficients of variations (CV) calculated. Errors and CV are calculated according to the following formula:

Error (%) = (|mean estimated volume - theoretical volume|/theoretical volume)*100

CV (%) = Standard deviation (µL)/ mean volumen (µL) * 100

Record the values obtained in the Reporting Sheet (TOXILINE-DSP.Co TEST-VALIDATION-REPORTING SHEET.xls).

Acceptance criteria:

Range	**Error (%)**	**CV (%)**
21 - 200 µL	<3	<1,5

In case of the pipettes not being correctly calibrated, follow the manufacturer's manual for instructions of calibration. If you like more information on the calibration and verification of pipettes check the manufacturer's manual or ask ZEU-INMUNOTEC.

APPENDIX II

Spiking procedure

The vials contain okadaic acid in an aqueous solution.

1. Shake the tube containing OA spiking solution by vortex for 20 seconds.
2. Add the OA spiking solution to the “BM+OA” sample with a 1 mL micropipette.
3. Add 500 µL of methanol (see paragraph 8.1) with a 1 mL micropipette (100 % v/v) to the OA spiking solution vial, shake by vortex for 20 seconds and add the content to the “BM+OA” sample with a 1 mL micropipette.
4. Add 24.5 mL of methanol (see paragraph 8.1) with a 25 mL glass pipette (100 % v/v) to the “BM+OA” sample and shake for 2 minutes by vortex. Proceed with the extraction procedure as described in paragraph 11.2.

Precaution: If the assay has to be repeated the whole spiking procedure needs to be repeated. New samples “BM” and “BM+OA” can be requested at ANFACO-CECOPESCA. OA spiking solution can be requested at ZEU-INMUNOTEC.