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EUROPEAN UNION REFERENCE LABORATORY FOR MARINE BIOTOXINS

EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS

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Foreword

This Standard Operating Procedure has been prepared by the Working Group LC-MS for lipophilic toxins of the European network of National Reference Laboratories (NRL) for Marine Biotoxins. Members of the Working Group: Belgium NRL, France NRL, Germany NRL, Ireland NRL, Italy NRL, The Netherlands NRL, Sweden NRL and United Kingdom NRL, coordinated by the European Union Reference Laboratory for Marine Biotoxins (EU-RL-MB, Spain).

Introduction

Lipophilic marine biotoxins can be accumulated in different molluscan shellfish presenting a health risk to humans if contaminated shellfish are consumed. To protect public health, monitoring programmes for marine biotoxins have been established in many countries for detecting the presence of these compounds in shellfish tissues. Four chemical groups of toxins are included in the lipophilic toxins group: okadaic acid (including dinophysistoxins), pectenotoxin, azaspiracid and yessotoxin group toxins.

The regulatory structure in the European Union (EU) includes a series of regulations for the control of lipophilic toxins. Thus, Regulation (EC) Nº 853/2004 [1], Annex III Section VII Chapter V, lays down the maximum levels for lipophilic toxins in bivalve molluscs before being placed on the market for human consumption: for okadaic acid, dinophysis and pectenotoxins together, 160 micrograms of okadaic acid equivalents per kilogram; for azaspiracid, 160 micrograms of azaspiracid equivalents per kilogram.

The Regulation (EU) No 786/2013 amending Annex III to Regulation (EC) No 853/2004 of the European Parliament and of the Council as regards the permitted limits of yessotoxins in live bivalve mollusks, lays down for yessotoxins, 3.75 milligram of yessotoxin equivalent per kilogram.

Regarding methodologies, the Commission Regulation (EU) No 15/2011 [2], amending Regulation (EC) No 2074/2005 [3], as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs, establishes the EU-RL LC-MS/MS method as the reference method for the detection of lipophilic toxins and used as matter of routine, both for the purposes of official controls at any stage of the food chain and own-checks by food business operators.

1. Purpose

The purpose of this Standard Operating Procedure (SOP) is to detail a protocol for the determination of the Okadaic Acid (OA), Pectenotoxin (PTX),

Azaspiracid (AZA) and Yessotoxin (YTX) group toxins using LC-MS/MS methodologies.

This method was validated under the coordination of the European Union Reference Laboratory for marine biotoxins (EU-RL) in an inter-laboratory validation study carried out by the Member States. For further information on the validation see Annex A.

The application of this procedure will allow direct quantitative determination of okadaic acid (OA), pectenotoxin 2 (PTX2), azaspiracid 1 (AZA1), and yessotoxin (YTX) by means of the reference standards commercially available.

Assuming an equal response factor, the procedure was validated by using OA for the indirect quantification of dinophysistoxin 1 (DTX1) and dinophysistoxin 2 (DTX2); likewise PTX2 was used for the indirect quantification of PTX1, AZA1 was used for the indirect quantification of AZA2 and AZA3; and YTX was used for the indirect quantification of homo YTX, 45 OH YTX and 45 OH homo YTX. This approach provided satisfactory results in the validation study. However, direct quantification using the own compound is advisable when new certified reference standard materials are available.

2. Scope

This method is applicable to the determination of the lipophilic marine biotoxins in different molluscan shellfish matrices, both fresh and cooked, such as mussels, clams, razor clams and cockles.

To be applied to different matrices or processed state of shellfish, each laboratory should address fulfillment of performance characteristics as part of the in house validation.

3. Principle

The method is based on the extraction of OA, PTX, AZA and YTX group toxins with 100% methanol from homogenised tissue. Extracts are then filtered and directly analysed by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) in order to investigate the presence of free OA, free DTX1 and free DTX2, PTX1, PTX2, AZA1, AZA2, AZA3, YTX, homo YTX, 45 OH YTX and 45 OH homo YTX [4]. To determine the total content of OA group toxins, an alkaline hydrolysis is necessary from methanolic extract prior to LC-MS/MS analysis with the aim of converting any acylated esters of OA and/or DTXs to the parent OA and/or DTX1 or DTX2 toxins [5]. After hydrolysis, extracts are filtered and analysed by LC-MS/MS. Chromatographic separation is performed by gradient elution.

4. Reagents

Use only reagents of recognized analytical grade. Solvents shall be of quality for HPLC analysis, unless otherwise specified. Water must be ultra-pure (milli-Q or similar). Commercially available solutions with equivalent properties to those listed may be used.

NOTE: Since the use of this method involves reagents harmful to health, appropriate precautionary measures must be followed to prevent inhalation and skin contact. Wear a lab coat and use where necessary gloves and safety glasses. Work should be conducted within an extractor hood or fume cupboard environment.

4.1 Chemicals and solvents

4.1.1 Acetonitrile, HPLC grade or Hypergrade for LCMS

4.1.2 Methanol, HPLC grade

4.1.3 Formic acid (98-100% purity)

4.1.4 Ammonium formate (≥99% purity)

4.1.5 Hydrochloric acid (37% purity)

4.1.6 Hydrochloric acid 2.5 M

Add 20 ml hydrochloric acid (4.1.5) to a 100 ml volumetric flask and made up to the mark with water. This solution is stored at room temperature and can be used for 3 months.

4.1.7 Sodium hydroxide (≥99% purity)

4.1.8 Sodium hydroxide 2.5 M

Dissolve 10 g sodium hydroxide (4.1.7) in 75 ml water in a 100 ml volumetric flask and made up to the mark with water. This solution is store at room temperature and can be used for 3 months.

4.1.9 Ammonia (25%)

4.1.10 Ammonium hydrogencarbonate (bicarbonate; ≥98% purity)

4.1.11 Ammonium hydroxide solution (>25 % or greater purity)

4.2 Chromatographic solvents

Each mobile phase should be filtered through a membrane filter (0.45 µm if a conventional HPLC method is used or 0.2 µm with ultra-fast liquid chromatography).

Examples of possible chromatographic conditions are indicated in this procedure. However, the operator will be able to use the conditions that deem more appropriate.

4.2.1 Acidic chromatographic conditions [6]

Mobile phase A: 100% water with 2 mM ammonium formate + 50 mM formic acid
e.g. Preparation 1000 ml: dissolve 128 mg ammonium formate (4.1.4) in water and transfer into a 1000 ml volumetric flask; add 1.9 ml formic acid (4.1.3) and made up to the mark with water.
This solution is stored at room temperature and can be used for 1 week.

Mobile phase B: 95% acetonitrile: 5% water with 2 mM ammonium formate + 50 mM formic acid
e.g. Preparation 500 ml: dissolve 64 mg ammonium formate (4.1.4) in 24.06 ml water into a 500 ml volumetric flask; add 944 µl formic acid (4.1.3) and made up to the mark with acetonitrile.
This solution is stored at room temperature and can be used for 1 week.

4.2.2 Basic chromatographic conditions (I) [7]

Mobile phase A: 0.05 v/v % ammonia in water (pH 11)
e.g. Preparation 1000 ml: add with a positive displacement pipette 0.5 ml ammonia (4.1.9) to 1000 ml water and mix.
This solution is stored at room temperature and can be used for 1 month.

Mobile phase B: 0.05 v/v % ammonia in 90% acetonitrile
e.g. Preparation 1000 ml: add with the help of graduated cylinders 900 ml acetonitrile (4.1.1) and 100 ml water in a 1000 ml bottle; add with a positive displacement pipette 0.5 ml ammonia (4.1.9) and mix.
This solution is stored at room temperature and can be used for 1 month.

4.2.3 Basic chromatographic conditions (II)

Mobile phase A: 100 % water + 2 mM ammonium bicarbonate, pH 11 e.g. Preparation 500 ml: dissolve 79 mg ammonium bicarbonate (4.1.10) in 30 ml water and add into a 500 ml volumetric flask; add 7.5

ml ammonium hydroxide (4.1.11) and made up to the mark with water. Check pH. This solution is stored at room temperature and can be used for 48 hours after preparation.

Mobile phase B: 90 % acetonitrile: 10 % water + 2 mM ammonium bicarbonate, pH 11
e.g. Preparation 500 ml: dissolve 79 mg ammonium bicarbonate (4.1.10) in 33 ml water and add into a 500 ml volumetric flask; add 17.5 ml ammonium hydroxide (4.1.11) and made up to the mark with acetonitrile. This solution is stored at room temperature and can be used for 48 hours after preparation.

4.3 Reference Materials

NOTE Certified Reference Materials and Solutions can be purchased from National Research Council Canada (NRC), Institute for Marine Biosciences,Halifax (http://www.nrc-cnrc.gc.ca/eng/programs/imb/crmp.html). This is an example for suitable products available commercially. This information is given for the convenience of users of this Standard Operating Procedure and other certified materials can be used if available and if they can be shown to lead to the same results.

4.3.1 Certified reference material with okadaic acid and dinophysistoxin 1 (CRM-DSP-MUS-b). Homogenate of mussel (*Mytilus edulis)* digestive gland with okadaic acid and dinophysistoxin.

4.3.2 Okadaic acid (CRM-OA-c). Standard solution of okadaic acid in methanol.

4.3.3 Pectenotoxin 2 (CRM PTX2). Standard solution of pectenotoxin 2 in methanol.

4.3.4 Azaspiracid 1 (CRM AZA1). Standard solution of azaspiracid 1 in methanol.

4.3.5 Yessotoxin (CRM YTX). Standard solution of yesotoxin in methanol.

4.3.6 Azaspiracid 2 (CRM AZA2). Standard solution of azaspiracid 2 in methanol.

4.3.7 Azaspiracid 3 (CRM AZA3). Standard solution of azaspiracid 3 in methanol.

4.4 Standard solutions

4.4.1 Stock standard solution

Ampoules containing different toxins used in the inter-laboratory validation study (4.3.2 to 4.3.5) are supplied with a certified concentration for each toxin. A certain volume of the reference standards is diluted with methanol (4.1.2) to the volume to get a stock multitoxin standard solution. Table 1 shows an example to prepare a multitoxin stock standard solution from the commercially reference materials available when the validation study was performed.

Table 1. Example to prepare a stock standard solution with a concentration level of 200 ng/ml for OA, PTX-2, and AZA-1 and of 500 ng/ml for YTX.

Reference standard	Certified concentration (µg/ml)	Volume (µl)	Solvent (µl)	Total volume (µl)	Final concentration (ng/ml)
OA NRC CRM-OA-c Lot 20070328	14.3	14	711	1000	200
PTX-2 NRC CRM-PTX2 Lot 20021127	8.6	23			
AZA-1 NRC CRM-AZA1 Lot 20060719	1.24	161			
YTX NRC CRM-YTX Lot 20060308	5.3	91			500

NOTE: Please check the concentration for different reference standard lots since the certified concentration may change.

4.4.2 Working standard solutions

A certain volume of the multitoxin stock standard solution (4.4.1) is diluted with methanol (4.1.2) to the volume to prepare multitoxin working standard solutions for the calibration curve. These solutions can be used for 1 week, being stored in a freezer (< -20ºC) when not in use. A longer storage time is allowed if the stability has been proven in the laboratory. Table 2 shows an example to prepare the working standard solutions for the calibration curve.

Table 2. Example to prepare the working standard solutions with a concentration range 3 ng/ml to 40 ng/ml for AO, PTX-2, and AZA-1 and 5 ng/ml to 100 ng/ml for YTX.

Stock standard solution (µl)	Solvent (µl)	OA, PTX2 and AZA1 concentration (ng/ml)	YTX concentration (ng/ml)	Calibration standard
15	985	3	5	Std1
30	970	6	15	Std2
50	950	10	25	Std3
100	900	20	50	Std4
150	850	30	75	Std5
200	800	40	100	Std6

5. Equipment

Use conventional laboratory material and equipment and, in particular, the following:

5.1 Analytical balance, accuracy to the nearest 0.1 mg

5.2 Balance, accuracy to the nearest 0.01 g

5.3 High-speed blender or homogeniser

5.4 Shaker (e.g. Vortex)

5.5 Ultra Turrax™

5.6 Centrifuge, up to 2000 g

5.7 Heat block or water bath, at 76ºC

5.8 Instruments for sample preparation, knives, spatulas, scissors, stainless steel sieve, plastic jars

5.9 Volumetric flask, 20 ml, 100 ml, 250 ml, 500 ml and 1000 ml

5.10 Adjustable automatic pipettes and graduated cylinders

5.11 50 ml polypropylene centrifuge tubes

5.12 Syringe or membrane filter, pore size 0.45 µm

5.13 HPLC autosampler vials

5.14 Syringe for filter system

5.15 Syringe or membrane filter, pore size 0.2 µm

5.16 Analytical reverse phase HPLC column:

Examples for pH range between 2 and 8 (acidic conditions):

BDS-Hypersil C8, 50 mm (length) x 2 mm (diameter), 3 µm particle size.

Examples for both acidic conditions and alkaline conditions (pH range: 1-12):
X-Bridge C18, 50 mm (length) x 2.1 mm (diameter), 2.5 µm particle size.
Acquity UPLC® BEH C18, 50 mm (length) x 2.1 mm (diameter), 1.7 µm particle size.
X-Bridge C18, 150 mm (length) x 3 mm (diameter), 5 µm or 3.5 µm particle size.
X-Bridge C18, 150 mm (length) x 2.0 mm (diameter), 3.5 µm particle size.

5.17 Liquid chromatograph, system able to analyse in gradient mode

5.18 Mass spectrometer, equipped with an ESI interface and able to analyse in tandem MS/MS

6. Procedure

6.1 Sample preparation

Raw samples have to be thoroughly cleaned outside of the shellfish with fresh water. Open by cutting adductor. Rinse inside with fresh water to remove sand and foreign material. Remove meat from shell by separating adductor muscles and tissue connecting at hinge. Do not use heat or anaesthetics before opening the shell. After removal from shellfish, drain tissues in a sieve to remove salt water. For representative sampling, at least 100-150 g of pooled tissue should be homogenized in a blender or homogenizer (5.3). Sub-samples from this homogenate can be taken immediately after blending, while still well mixed, or after mixing again.

6.2 Extraction procedure

Accurately weigh 2.00 g ± 0.05 g of tissue homogenate into a centrifuge tube (5.11). Add 9.0 ml of 100% methanol (4.1.2) and homogenize the sample via vortex mixing (5.4) for 3 min at maximum speed level. Centrifuge at 2000 *g* or higher for 10 min at approx. 20ºC (5.6) and transfer the supernatant to a 20 ml volumetric flask (5.9). Repeat the extraction of the residual tissue pellet with another 9.0 ml of methanol 100% (4.1.2) and homogenize for 1 min in Ultra Turrax™ (5.5). After centrifugation (at 2000 g or higher for 10 min and approx. 20ºC), transfer and combine the supernatant with the first extract and make up the extract to 20 ml with 100% methanol (4.1.2).

6.3 Free OA, PTX, AZA and YTX group toxins analysis

The determination of free OA, PTX, AZA and YTX group toxins is performed after filtering an aliquot of the methanolic extract (6.2) through a dry methanol-compatible 0.45 µm or 0.2 µm syringe filter and injecting between 5 µl and 20 µl, depending on sensitivity of instrument, onto LC-MS system.

6.4 Hydrolysis

In order to detect and quantify the total content of OA/DTX toxins an alkaline hydrolysis is required before LC-MS/MS analysis.

The hydrolysis consists of adding NaOH 2.5 M (4.1.8) to an aliquot of the methanolic extract (6.2), homogenize in vortex for 0.5 minutes and heat the mixture at 76 ºC for 40 minutes. Then, cool to room temperature, neutralise with HCl 2.5 M (4.1.6) and homogenise in vortex for 0.5 minutes. Filter this extract through a dry methanol-compatible 0.45 µm or 0.2 µm syringe filter and, depending on sensitivity of MS instrument used for the analysis, inject between 5 µl and 20 µl onto LC column.

A ratio of extract to base-acid of 125 (µl base-acid per ml extract) is required.

The following procedures are examples:

1.- In a test tube, add 313 µl of NaOH 2.5 M (4.1.8) to 2.5 ml of methanolic extract (6.2), homogenise using a vortex mixer for 0.5 minutes and heat the mixture using a heating block or water bath set at 76 ºC for 40 minutes. Cool to room temperature, neutralise with 313 µl of HCl 2.5 M (4.1.6) and homogenise in vortex for 0.5 minutes. Filter this extract through a dry methanol-compatible 0.45 µm or 0.2 µm syringe filter and inject 5 µl -20 µl onto the LC column.
2.- In a HPLC vial, add 125 µl of NaOH 2.5 M (4.1.8) to 1.0 ml of methanolic extract (6.2), homogenise using a vortex mixer for 0.5 minutes and heat the mixture using a heating block or water bath set at 76 ºC for 40 minutes. Cool to room temperature, neutralise with 125 µl of HCl 2.5 M (4.1.6) and homogenise in vortex for 0.5 minutes. Filter this extract through a dry methanol-compatible 0.45 µm or 0.2 µm syringe filter and inject 5 µl -20 µl onto the LC column.
NOTE: During hydrolysis vials have to be firmly closed (boiling point of methanol is 65 ºC). By weighting the tube or vials before and after heating, it can be checked if there was methanol evaporation during the process. If methanol evaporation is observed, the volume must be completed with methanol to the weight before continuing the process.

6.5 Concentration step

If necessary, a concentration step should be used in order to achieve a lower limit of quantification (with a signal-to-noise ratio of 10:1) of 40 µg/kg for OA and AZA1, of 50 µg/kg for PTX2 and 60 µg/kg for YTX.

6.6 Clean-up

To be used, if necessary, to eliminate matrix effects. Possible options: liquid-liquid partitioning, SPE, etc. If this approach is used, the recovery of this step must be individually evaluated and reported by the laboratory.

7. LC-MS/MS determination

7.1 LC conditions

Chromatographic conditions are not set; they may be adjusted to the respective laboratory conditions. Analytes that cannot be distinguished by mass spectrometry must be separated by means of chromatography (e.g. OA and DTX2).

The selected chromatographic conditions must be reported.

Based on pH of mobile phase, some possible options could be:

7.1.1 LC measuring conditions (chromatography under acidic conditions)

Tables 3A and 3B shows chromatographic conditions proved to be suitable under acidic conditions (4.2.1).

Table 3A. Possible LC conditions for the analysis of lipophilic toxins for a C8 column under acidic conditions:

Column	BDS-Hypersil C8, 50 mm (length) x 2 mm (diameter), 3 µm particle size		
Flow	0.2 ml/min		
Injection volume	5 µl -10 µl (depending on MS sensitivity)		
Column temp.	25-40 °C		
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0	70	30
	8	10	90
	11	10	90
	11.5	70	30
	17	70	30

Table 3B. Possible LC conditions for the analysis of lipophilic toxins for a C18 column under acidic conditions:

Column	X-Bridge C18, 50 mm (length) x 2.1 mm (diameter), 2.5 µm particle size		
Flow	0.3 ml/min		
Injection volume	5 µl -20 µl (depending on MS sensitivity)		
Column temp.	25°C		
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0	90	10
	4	20	80
	6	20	80
	6.5	90	10
	9	90	10

7.1.2 LC measuring conditions (chromatography under basic conditions)

Tables 4A and 4B shows chromatographic conditions proved to be suitable under basic conditions.

Table 4A. Possible LC conditions for the analysis of lipophilic toxins for a C18 column under basic conditions (4.2.2):

Column	X-Bridge C18, 150 mm (length) x 3 mm (diameter), 5 µm or 3.5 µm particle size		
Flow	0.4 ml/min		
Injection volume	10 µl		
Column temp.	40 °C		
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0	90	10
	1	90	10
	10	10	90
	13	10	90
	15	90	10
	19	90	10

Table 4B. Possible LC conditions for the analysis of lipophilic toxins for a C18 column under basic conditions (4.2.3):

Column	X-Bridge C18, 150 mm (length) x 2.0 mm (diameter), 3.5 µm particle size		
Flow	0.3 ml/min		
Injection volume	5 µl -10 µl		
Column temp.	30 °C		
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0	75	25
	1	75	25
	11.4	0	100
	16.7	0	100
	17	75	25
	22.5	75	25

7.2 Mass spectrometric detection

Before sample analysis, the mass spectrometric (MS) parameters should have been previously optimised with toxin standards in order to achieve the maximum level of sensitivity in the analysis. These parameters depend on the instrument model.

If no individual reference standards are available, MS parameters have to be based on those optimised for the available standards. Consequently, the same DP[V], CE[eV] and dwell time values have to be used for the detection of each

toxin group (OA, PTX, AZA and YTX) since each group have the same fragmentation pathway.

Each chromatographic peak should be defined by a range of at least 10 to 15 data points per peak for an accurate description of the peak.

The MS detection has to perform using two transitions per toxin. The transition with the highest intensity is used for quantification, while the transition with the lowest intensity is used for confirmatory purposes.

Table 5. Example of source parameters for a 3200 QTrap LC/MS/MS system (Applied Biosystem/MDS SCIEX)

	AO, PTX, AZA group	**YTX group**
Curtain Gas (CUR)	20 psi	10 psi
Collision Gas (CAD)	Medium	Medium
Voltage (IS)	4500 v	4500 v
Temperature (TEM)	650 °C	600 °C
Gas 1 (GS1)	40 psi	50 psi
Gas 2 (GS2)	60 psi	50 psi

Table 6. Example of MS/MS fragmentation conditions for a 3200 QTrap LC/MS/MS system (Applied Biosystem/MDS SCIEX)

Compound	**ESI**	**Q1**	**Q3**	**mseg**	**DP(v)**	**EP(v)**	**CEP(v)**	**CE(v)**	**CXP(v)**
OA	NEG	803.5	255.0	125	-120	-10	-28	-62	-2
OA	NEG	803.5	113.0	125	-120	-10	-28	-60	-2
DTX-2	NEG	803.5	255.0	125	-120	-10	-28	-62	-2
DTX-2	NEG	803.5	113.0	125	-120	-10	-28	-60	-2
DTX-1	NEG	817.5	255.0	125	-120	-10	-28	-62	-2
DTX-1	NEG	817.5	113.0	125	-120	-10	-28	-60	-2
YTX	NEG	1141.5	1061.7	150	-60	-12	-38	-46	-8
YTX	NEG	1141.5	855.5	150	-60	-12	-38	-108	-10
45 OH-YTX	NEG	1157.5	1077.7	150	-60	-12	-38	-46	-8
45 OH-YTX	NEG	1157.5	871.5	150	-60	-12	-38	-108	-10
HomoYTX	NEG	1155.5	1075.5	150	-60	-12	-38	-46	-8
HomoYTX	NEG	1155.5	869.5	150	-60	-12	-38	-108	-10
45 OH-HomoYTX	NEG	1171.5	1091.5	150	-60	-12	-38	-46	-8
45 OH-HomoYTX	NEG	1171.5	869.5	150	-60	-12	-38	-108	-5
PTX-1	POS	892.5	821.5	35	66	10	100*	39	8
PTX-1	POS	892.5	213.2	35	66	10	100*	51	4
PTX-2	POS	876.5	823.4	35	66	10.5	56	39	8
PTX-2	POS	876.5	213.2	35	66	10.5	56	51	4
AZA-1	POS	842.5	824.5	35	81	4.5	64	55	6
AZA-1	POS	842.5	806.5	35	81	4.5	64	55	6
AZA-2	POS	856.5	838.5	35	81	4.5	76*	55	8
AZA-2	POS	856.5	820.5	35	81	4.5	76*	55	8
AZA-3	POS	828.5	810.5	35	81	4.5	68*	55	6
AZA-3	POS	828.5	792.5	35	81	4.5	68*	55	6

* If no individual toxins available, values have to be based on those optimised for the available standards (usually PTX2 for PTX group and AZA1 for AZA group).

8. Calibration and quantification

8.1 Calibration curve and sample injection

Prepare a calibration curve each day of analysis (4.4.2).

The following sequence of injection should be used for sample analysis:

1. One injection of each calibration curve level (first set) commencing with the lowest concentration to the highest concentration;
2. one injection of the procedural blank (Blank QC), prepared during extraction of real samples;
3. sample extracts by duplicate injection including positive QC (intermediate calibration standard, spiked extract, CRM);
4. one injection of the procedural blank (Blank QC);
5. second injection of each calibration curve level (second set).

The *intra*-batch, response drift, defined here as the variation between calibration slopes between the first and second sets of calibration standards should not be ≥ 25 %.

NOTE: Although this procedure has been validated by using duplicate injection of samples, this could not be practical for routine analysis where a large number of samples need to be analysed. Each laboratory must check within laboratory repeatability if single injection of sample is to be used.

To carry out the toxin determination, plot peak area against the concentration of the injected calibration solution. Ensure that the slope of the calibration curve shows a linear regression. The correlation coefficient, r^2, has to be ≥ 0.98. Integrate peak areas of each detected toxin in each sample injection and determine the average peak areas for each sample.

The procedural blank (Blank QC) will be methanol for free OA, PTX, AZA and YTX groups analysis and methanol after hydrolysis as described in 6.4 for total OA group analysis. In this blank QC no toxins should be detected (< LOD or < 10% of the lowest calibration point).

8.2 Identification and confirmation

Identify the presence of each toxin, with the reference standards available, by comparing the retention time of the analytes in the sample with those of the standards. An analyte is detected when the deviation between the retention

time of the analyte and the standards not exceed 3%.

For a correct identification, a good baseline separation between OA and its isomer DTX2 must be assured. Peak resolution between OA/DTX2 can be calculated using the following expression:

$$R_s = \frac{2x(RT_2 - RT_1)}{(W_1 + W_2)}$$

Where RT1 and RT2 and W1 and W2 are, respectively, the times and widths at the baseline of the peaks of the two immediately adjacent peaks (RT2 > RT1). An acceptable resolution between two peaks is considered to be Rs > 1.0, and complete baseline resolution has Rs > 1.5. If Rs < 1.0 chromatographic conditions should be adjusted.

For confirmatory purposes of each identified toxin, the signal-to-noise (S/N) of the product ion with the lowest intensity should be ≥ 3.

For quantification, the transition with the highest intensity is used.

The ratio between the two ions (quantifier and qualifier) must be recorded.

8.3 Quantification

The quantification of each toxin is determined using the external standard calibration method. According to the approach followed in the interlaboratory validation study of the method and assuming an equi-molar response, the calibration curve constructed for OA is to be used also for the quantification of DTX1 and DTX2; the calibration curve constructed for PTX2 is to be used also for PTX1, the one for AZA1 is to be used also for quantification of AZA2 and AZA3, and the calibration curve for YTX is to be used also for quantification of homo YTX, 45 OH YTX and 45 OH homo YTX. This approach can be changed when new certified reference standards are available.

Evaluation is based on the linear equation of the regression line of the individual toxins with standards available. When the signal for a toxin in the analysed sample is higher than the signal of the highest calibration standard, the extract must be diluted with methanol to get a signal within the calibration curve and the dilution factor (D) should be taken into account for calculations.

Therefore, from the calibration curve, the concentration of the individual toxins in each analysed sample is calculated using the following equation:

$$\textbf{Concentration (µg toxin/kg)} = \left(\frac{y-b}{a}\right) \boldsymbol{x} \frac{\frac{V_F(ml)}{V_H(ml)} \times V_T(ml)}{W(g)} \times D$$

where:

y = Area of the chromatographic peak

b = intercept of the regression linear

a = slope of the calibration curve

V_T= Total volume of crude extract (20 mL)

V_H=Volume of extract used for performing the hydrolysis.

V_F= Final volume of extract after hydrolysis (and clean-up / concentration)

W= Sample tissue weigh (2 g)

D= Dilution factor (if extract has been diluted)

8.4 Recovery correction and matrix correction

With the aim of evaluating effects of the procedure and of the matrix, a reference material or a spiked extract can be used for toxin recovery or matrix correction.

NOTE This Standard Operating Procedure has been interlaboratory validated using non-corrected and corrected results. For OA group toxins determination, correction using certified reference material generally improves performance characteristics (Annex A). Each laboratory must assess matrix effects on their instrumentation and determine if correction is necessary.

For OA group toxins determination, the CRM-DSP-Mus-b can be used for toxin recovery correction. The following preparation of this certified reference material was found to be appropriate:

1. transferring to a 50 ml centrifuge tube, 1.9 g of homogenate from the CRM bottle (this amount should be weighed accurately after thoroughly vortex mixing the total content of the bottle);

2. double extract with methanol 100% following the same procedure indicated in point 6.2;

3. dilute (1/50) the CRM crude extract by using a calibrated pipette, transferring 400 µl of crude extract to 20 ml series A volumetric flask and making up to the 20 ml mark with 100% MeOH;

4. dilute (1/6) the CRM crude extract by using a calibrated pipette, transferring 3300 µl of crude extract to another 20 ml series A volumetric flask and making up to the 20 ml mark with 100% MeOH.

Table 7 shows the expected concentration (assuming 100% recovery of each toxin after extraction) of each dilution of CRM extract based on the certified values.

Table 7. CRM Mus-b concentration for OA group toxins recovery correction.

NRC CRM-DSP-MUS-b Lot 200304	**Expected concentration (ng/ml) using a dilution factor of 1/6**	**Expected concentration (ng/ml) using a dilution factor of 1/50**
OA	na	19.2 (equivalent to 192 µg/kg)
DTX-1	20.4 (equivalent to 204 |µg/kg)	na

NOTE: Please check the concentration for reference material lots since the certified concentration may change.

When a reference material (recovery for the procedure) or a spiked extract (matrix effect) is used for correction, the recovery values obtained in the analysis of the CRM or spiked extract will be used according to the following expression:

Corrected concentration:

µg toxin/kg = $(\mu g/kg)_{EXTERNAL\ \mathrm{CALIBRATION}}\ x\ \dfrac{100}{\%R_{\mathrm{CRMorspikedextract}}}$

where:

$(\mu g/kg)_{EXTERNAL\ CALIBRATION}$: concentration calculated by external calibration according to point 8.3

$\%R_{CRM}$: recovery obtained in the analysis of reference material or spiked extract, $\Rightarrow$

$$\%R = \frac{(ng/ml)_{CALCULATED}}{(ng/ml)_{THEORETICAL}} \times 100$$

9. Expression of results

To express results by toxin group according to European legislation, as µg equivalents/kg or mg equivalents/kg, the use of the Toxicity Equivalent Factors (TEFs) indicated in table 8 is required, as adopted by the Scientific Panel on Contaminants in the Food Chain of the European Food Safety Authority (EFSA) [8].

Table 8. TEFs adopted by EFSA for regulated lipophilic marine biotoxins.

Toxin group	Analogue	TEF	Results expression
OA group	OA	1	µg OA equivalents/kg
	DTX1	1	
	DTX2	0.6	
PTX group	PTX2	1	µg PTX equivalents/kg
	PTX1	1	
AZA group	AZA1	1	µg AZA equivalents/kg
	AZA2	1.8	
	AZA3	1.4	
YTX group	YTX	1	mg YTX equivalents/kg
	homo YTX	1	
	45 OH YTX	1	
	45 OH homo YTX	0.5	

Therefore, after calculating the individual content of each toxin/analogue, it should be multiplied by the TEF before summing the total equivalents for the respective group toxins.

10. Quality control criteria

Table 9 summarizes the criteria that the quality control (QC) parameters set in the procedure have to fulfil for the quantitative analysis of lipophilic marine biotoxins.

Table 9. Quality control criteria for acceptance the quantitative analysis of lipophilic marine biotoxins.

QC parameter	Criterion
Chromatographic resolution	Peak resolution OA/DTX2 **>** 1.0
Sensitivity	S/N of the product ion with the lowest intensity ≥ 3
Calibration curve	Correlation coefficient r^2 ≥ 0.98 derived from at least five calibration points and either constructed as the mean of the first and second set of the calibration curve injected as described in 8.1
Response drift	25% slope variation between the two sets of the calibration curve
Blank QC	To be injected after high standard of calibration curve and after samples as described 8.1. No signal for lipophilic toxins (< LOD or < 10% of the lowest calibration point)
Retention time (RT) drift	< 3%

Annex A. Performance characteristics

The following data were obtained in an interlaboratory validation study organised by the European Union Reference Laboratory for marine biotoxins (EU-RLMB), with the assessment of the advice and collaboration of the Working Group LC-MS for lipophilic toxins of the European network of National Reference Laboratories (NRL) for Marine Biotoxins.

The purpose of the validation study was to determine accuracy, repeatability and between-laboratory reproducibility of the method describes in the “EU-Harmonised Standard Operating Procedure (SOP) for determination of Lipophilic marine biotoxins in molluscs using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)”. The study was carried out in two phases: in 2009, the SOP was evaluated for the determination of OA group toxins and during a second phase in 2010, the evaluation was also extended to AZA group toxins, PTX group toxins and YTX group toxins.

10 materials (5 of them as blind duplicates) were tested by 12 participants during the first phase of the study. In the second phase of the study, 7 materials (as blind duplicates) and a pre released Reference Material were tested by 15 laboratories from 10 European countries. Materials included different species of molluscs (mussels, cockles, clams and razor clams) with fortified, naturally contaminated at different levels or blanks for the different lipophilic marine biotoxins groups evaluated.

Annex A-Table 1. Performance characteristics for **OA** determination obtained from the 2009 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of OA-Group Toxins by LC-MS/MS". Recovery corrected results.

Material	N°of labs a(b)	Mean[(1)] µg/kg	Recovery,%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
CRM diluted with blank mussel	8(0)	39.4[(1)]	71.3	-	11.9	0.46
CRM diluted with blank mussel	8(0)	70.6[(1)]	89.4	-	13.9	0.58
CRM diluted with blank mussel	8(0)	150.4[(1)]	84.1	-	17.3	0.81
CRM diluted with blank mussel	8(0)	224 2[(1)]	83.6	-	15.3	0.76
Naturally contaminated precooked mussel	11(1)	110.6[(2)]	-	7.9	33.1	1.48
Naturally contaminated raw cockle	10(2)	155.2[(2)]	-	7.5	18.4	0.87
Naturally contaminated precooked mussel	12(0)	202.4[(2)]	-	7.0	26.3	1.29
Naturally contaminated raw clam	11(1)	72.2[(2)]	-	8.8	25.9	1.09

a= Number of laboratories remaining after removal of the number of outliers indicated by (b) [(1)] Free OA [(2)] Total OA

Annex A -Table 2. Performance characteristics for **DTX1** determination obtained from the 2009 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of OA-Group Toxins by LC-MS/MS". Recovery corrected results.

Material	N°of labs a(b)	Mean[(1)] µg/kg	Recovery,%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
CRM diluted with blank mussel	6(0)	35.3[(1)][(3)]	114.3	-	17.8	0.67
Naturally contaminated precooked mussel	11(0)	58.0[(2)]	-	16.4	30.1	1.23

a= Number of laboratories remaining after removal of the number of outliers indicated by (b).
[(1)] Free DTX1 [(2)] Total DTX1 [(3)] Evaluation of performance characteristics was carried out only with six valid results since some participants has not enough sensibility to quantify DTX1 at this level

Annex A -Table 3. Performance characteristics for **DTX2** determination obtained from the 2009 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of OA-Group Toxins by LC-MS/MS". Recovery corrected results.

Material	N°of labs a(b)	Mean µg/kg	Recovery,%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
Naturally contaminated raw	11(0)	131.1	-	7.2	42.9	1.97

a= Number of laboratories remaining after removal of the number of outliers indicated by (b)

Annex A -Table 4a. Performance characteristics for **OA** determination obtained from the 2010 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of marine lipophilic biotoxins in molluscs by LC-MS/MS". Uncorrected results.

Material	N°of labs a(b)	Mean µg/kg	Recovery,%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
Donax trunculus (wedge shell clam)	15(0)	184	-	10.8	28.6	1.39
Ensis arcuatus (razor clam)	12(3)	84.6	-	12.4	32.7	1.41
Mytilus edulis (raw mussel)	13(1)	86.0	-	12.5	31.2	1.35
Chamelea gallina (stripped venus)	14(1)	182	-	8.34	34.1	1.65
Mytilus edulis (cooked mussel)	15(0)	431	-	4.87	35.5	1.95
Cerastoderma edule (cockle)	15(0)	154	-	22.2	39.7	1.87
Mytilus edulis (cooked mussel)	13(2)	338	-	4.38	32.3	1.72

a= Number of laboratories remaining after removal of the number of outliers indicated by (b)

Annex A -Table 4b. Performance characteristics for **OA** determination obtained from the 2010 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of marine lipophilic biotoxins in molluscs by LC-MS/MS". Recovery corrected results (CRM-DSP-Mus-b).

Material	N°of labs a(b)	Mean µg/kg	Recovery,%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
Donax trunculus (wedge shell clam)	14(0)	175	-	10.8	26.1	1.26
Ensis arcuatus (razor clam)	12(2)	83.6	-	19.0	37.3	1.61
Mytilus edulis (raw mussel)	12(1)	80.0	-	12.7	31.3	1.34
Chamelea gallina (stripped venus)	12(2)	175	-	9.87	16.3	0.79
Mytilus edulis (cooked mussel)	12(2)	406	-	5.32	13.1	0.71
Cerastoderma edule (cockle)	12(2)	127	-	12.2	36.1	1.65
Mytilus edulis (cooked mussel)	12(2)	311	-	4.07	31.4	1.65

a= Number of laboratories remaining after removal of the number of outliers indicated by (b)

Annex A -Table 5a. Performance characteristics for **DTX2** determination (against OA calibrant) obtained from the 2010 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of marine lipophilic biotoxins in molluscs by LC-MS/MS". Uncorrected results.

Material	N°of labs a(b)	Mean µg/kg	Recovery,%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
Donax trunculus (wedge shell clam)	14(1)	238	-	8.80	27.4	1.38
Ensis arcuatus (razor clam)	11(2)	68.2	-	14.9	23.2	0.97
Chamelea gallina (stripped venus)	14(0)	80.4	-	19.7	34.4	1.47
Mytilus edulis (cooked mussel)	11(1)	57.7	-	8.03	34.5	1.40
Cerastoderma edule (cockle)	15(0)	104	-	21.9	33.1	1.47
Mytilus edulis (cooked mussel)	14(1)	400	-	6.32	32.3	1.76

a= Number of laboratories remaining after removal of the number of outliers indicated by (b)

Annex A -Table 5b. Performance characteristics for **DTX2** determination (against OA calibrant) obtained from the 2010 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of marine lipophilic biotoxins in molluscs by LC-MS/MS". Recovery corrected results (CRM-DSP-Mus-b).

Material	N°of labs a(b)	Mean µg/kg	Recovery,%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
Donax trunculus (wedge shell clam)	13(1)	228	-	8.44	25.7	1.29
Ensis arcuatus (razor clam)	12(1)	69.8	-	13.8	33.2	1.39
Chamelea gallina (stripped venus)	12(2)	73.2	-	10.8	26.1	1.10
Mytilus edulis (cooked mussel)	11(1)	52.7	-	8.25	32.6	1.31
Cerastoderma edule (cockle)	14(0)	98.2	-	24.1	40.7	1.80
Mytilus edulis (cooked mussel)	12(2)	350	-	5.73	27.6	1.47

a= Number of laboratories remaining after removal of the number of outliers indicated by (b)

Annex A -Table 6a. Performance characteristics for **DTX1** determination (against OA calibrant) obtained from the 2010 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of marine lipophilic biotoxins in molluscs by LC-MS/MS". Uncorrected results.

Material	N°of labs a(b)	Mean µg/kg	Recovery,%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
Mytilus edulis (raw mussel)	14(1)	422	-	5.84	31.0	1.70
Mytilus edulis (cooked mussel)	15(0)	273	-	6.66	39.2	2.01

a= Number of laboratories remaining after removal of the number of outliers indicated by (b)

Annex A -Table 6b. Performance characteristics for **DTX1** determination (against OA calibrant) obtained from the 2010 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of marine lipophilic biotoxins in molluscs by LC-MS/MS". Recovery corrected results (CRM-DSP-Mus-b).

Material	N°of labs a(b)	Mean µg/kg	Recovery,%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
Mytilus edulis (raw mussel)	13(1)	312	-	8.86	16.4	0.86
Mytilus edulis (cooked mussel)	12(2)	205	-	6.53	21.5	1.06

a= Number of laboratories remaining after removal of the number of outliers indicated by (b)

Annex A -Table 7a. Performance characteristics for **AZA1** determination obtained from the 2010 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of marine lipophilic biotoxins in molluscs by LC-MS/MS". Uncorrected results.

Material	N°of labs a(b)	Mean µg/kg	Recovery,%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
Donax trunculus (wedge shell clam)	13(1)	80.4	77.9	11.5	16.9	0.72
Ensis arcuatus (razor clam)	13(1)	47.8	71.8	12.0	23.9	0.94
Mytilus edulis (raw mussel)	13(1)	347	-	10.2	25.8	1.38
Chamelea gallina (stripped venus)	13(2)	109	71.4	5.14	19.9	0.89
Mytilus edulis (cooked mussel)	14(1)	231	-	3.85	28.9	1.45
Cerastoderma edule (cockle)	12(2)	40.7	73.7	6.55	14.5	0.56
Mytilus edulis (cooked mussel)	14(1)	434	-	5.43	23.1	1.27

a= Number of laboratories remaining after removal of the number of outliers indicated by (b)

Annex A -Table 7b. Performance characteristics for **AZA1** determination obtained from the 2010 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of marine lipophilic biotoxins in molluscs by LC-MS/MS". Recovery corrected results.

Material	N°of labs a(b)	Mean µg/kg	Recovery,%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
Donax trunculus (wedge shell clam)	14(0)	91.5	88.7	14.8	28.7	1.25
Ensis arcuatus (razor clam)	13(1)	50.5	75.9	12.6	17.8	0.71
Mytilus edulis (raw mussel)	10(4)	323	-	6.66	10.7	0.57
Chamelea gallina (stripped venus)	14(1)	113	74.0	5.79	17.9	0.80
Mytilus edulis (cooked mussel)	14(1)	225	-	3.99	15.2	0.76
Cerastoderma edule (cockle)	13(1)	43.5	78.8	11.0	17.3	0.68
Mytilus edulis (cooked mussel)	12(1)	472	-	3.83	12.8	0.71

a= Number of laboratories remaining after removal of the number of outliers indicated by (b)

Annex A -Table 8. Performance characteristics for **AZA2** determination (against AZA1 calibrant) obtained from the 2010 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of marine lipophilic biotoxins in molluscs by LC-MS/MS". Uncorrected results.

Material	N°of labs a(b)	Mean µg/kg	Recovery[1],%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
Donax trunculus (wedge shell clam)	10(1)	30.3	138.4	12.1	20.4	0.76
Ensis arcuatus (razor clam)	11(2)	39.5	136.2	6.67	28.6	1.10
Mytilus edulis (raw mussel)	12(1)	84.4	-	10.2	25.1	1.08
Chamelea gallina (stripped venus)	11(3)	63.2	117.7	6.91	25.2	1.04
Mytilus edulis (cooked mussel)	13(2)	62.1	-	4.92	28.4	1.17
Cerastoderma edule (cockle)	12(2)	40.0	117.6	9.25	24.2	0.93
Mytilus edulis (cooked mussel)	11(4)	111	-	1.70	29.2	1.31

a= Number of laboratories remaining after removal of the number of outliers indicated by (b)
[1]Reference concentration from EURLMB analysis (n=20) using a validated and accredited methodology

Annex A -Table 9. Performance characteristics for **AZA3** determination (against AZA1 calibrant) obtained from the 2010 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of marine lipophilic biotoxins in molluscs by LC-MS/MS". Uncorrected results.

Material	N°of labs a(b)	Mean µg/kg	Recovery,%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
Mytilus edulis (raw mussel)	8(2)	30.1	-	12.0	42.6	1.57
Mytilus edulis (cooked mussel)	12(3)	95.3	-	13.3	25.4	1.12
Mytilus edulis (cooked mussel)	12(3)	106	-	7.68	20.4	0.91

a= Number of laboratories remaining after removal of the number of outliers indicated by (b)

Annex A -Table 10a. Performance characteristics for **PTX2** determination obtained from the 2010 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of marine lipophilic biotoxins in molluscs by LC-MS/MS". Uncorrected results.

Material	N°of labs a(b)	Mean µg/kg	Recovery[1],%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
Donax trunculus (wedge shell clam)	13(0)	82.9	114	12.6	32.8	1.41
Chamelea gallina (stripped venus)	13(0)	77.7	72.8	9.86	34.0	1.45

a= Number of laboratories remaining after removal of the number of outliers indicated by (b).
[1]Reference concentration from EURLMB analysis (n=20) using a validated and accredited methodology

Annex A -Table 10b. Performance characteristics for **PTX2** determination obtained from the 2010 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of marine lipophilic biotoxins in molluscs by LC-MS/MS". Recovery corrected results.

Material	N°of labs a(b)	Mean µg/kg	Recovery[1],%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
Donax trunculus (wedge shell clam)	13(0)	90.4	124	10.5	38.6	1.68
Chamelea gallina (stripped venus)	12(1)	76.7	71.9	8.52	28.6	1.21

a= Number of laboratories remaining after removal of the number of outliers indicated by (b).
[1]Reference concentration from EURLMB analysis (n=20) using a validated and accredited methodology

Annex A -Table 11a. Performance characteristics for **YTX** determination obtained from the 2010 Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of marine lipophilic biotoxins in molluscs by LC-MS/MS". Uncorrected results.

Material	N°of labs a(b)	Mean µg/kg	Recovery,%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
Mytilus edulis (raw mussel)	11(0)	111	-	9.16	35.2	1.58

a= Number of laboratories remaining after removal of the number of outliers indicated by (b).

Annex A -Table 11b. Performance characteristics for **YTX** determination obtained from the Interlaboratory Validation Study of the "EU-Harmonised Standard Operating Procedure for determination of marine lipophilic biotoxins in molluscs by LC-MS/MS". Recovery corrected results.

Material	N°of labs a(b)	Mean µg/kg	Recovery,%	Repeatability RSD_r %	Reproducibility	
					RSD_R %	HorRat
Mytilus edulis (raw mussel)	11(0)	104	-	9.19	29.3	1.30

a= Number of laboratories remaining after removal of the number of outliers indicated by (b)

Annex B. EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS: technical issues

During the Single-Laboratory Validation [6] and Accreditation Process carried out at the EU-RL-MB with the "EU- Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS", the following technical issues were found to be important and are presented to support laboratories during the implementation, validation and accreditation of this Standard Operating Procedure:

ISSUE	SUMMARY	COMMENTS
Quantification	EU-RL LC-MS/MS method has been validated in an inter-laboratory study using the available tools when that validation was performed. Due to the absence of certified reference material for all lipophilic toxins regulated in the EU legislation, the approach of assuming equi-molar response among toxin with certified reference standard and toxins belonging to the same toxin group was used.	The assumption of an equal response factor for toxin quantification provided satisfactory results in the interlaboratory validation. However, direct quantification using the own compound is advisable when new reference standard materials are available.
Sample injection	EU-RL LC-MS/MS method has been validated by using duplicate injection.	When Single Lab Validation is performed, each laboratory must check within laboratory repeatability if single injection of sample is to be used.

ISSUE	SUMMARY	COMMENTS
Recovery correction	EU-RL LC-MS/MS method has been validated using non-corrected and corrected results.	When Single Lab Validation is performed, each laboratory must assess matrix effects on their instrument and determine if correction is necessary. Reference material or spiked extract can be used for toxin recovery or matrix correction if necessary. The approach used for each laboratory has to be perfectly proved through in-house validation experiments.
Identification	Identification of each toxin is performed by comparing the retention time of the analytes in the sample with those of the reference standards when available.	It is advisable using relative retention time for identification of those toxins for which there is no reference standard available.
Retention time drift	Based on the EU-RL LC-MS/MS method, a retention time drift < 3% is allowed	However, if a RT drift of >2% is occurring then LC problems could exist.
Confirmation	No criteria for toxin confirmation has been included in the EU-RL LC-MS/MS method	Results obtained in the SLV performed by the EURLMB (Villar-González et al., 2011. J. of AOAC Int., 94(3), 909-922) have proved the usefulness of approach described in Commission Decision 2002/657/EC for confirmation purposes. In order to apply this criterion, the second ion/transition should be detected and present with a signal-to-noise ratio of ≥3:1 within all working range.
Hydrolysis	In order to quantify the total content of OA/DTX toxins an alkaline hydrolysis is required before LC-MS/MS analysis.	By weighting the tube or vials before and after heating, it should be checked if there was methanol evaporation during the process. If methanol evaporation is observed, the volume must be completed with methanol to the weight before continuing the process.

ANNEX C: Procedure for the extraction of lipophilic toxins from processed mussels*

This Annex provides details on the procedure for the extraction of lipophilic toxins from processed mussels.

This procedure has been discussed and agreed by the EURL/NRLs Working Group of LC-MS/MS, following the EURLMB proposal, which has been also discussed with the EU Commission (DGSANCO) and presented at the Working Group on bivalve mussels in Grange (Ireland) (Dec 11-12 2014)

Procedure for the extraction of lipophilic toxins from processed mussels*.

During processing there is a loss of water due to steaming and or autoclaving. On average steaming will result in the loss of 30% water and autoclaving 50%. In order to correct for this loss of water and assist with homogenisation and extraction this water should be added to the processed mussels before testing. This is necessary if the determined toxin concentration is to be related to the regulatory limit which is set for live bivalve molluscs.

1. **Canned mussels**

   a. packed in oil, sauce, broth and water:
   Follow AOAC Official Method 937.07 [9](c). If the ratio solid/liquid is high (i.e. > 50/50) and/or a heterogenic slurry is obtained add water. Take this dilution factor into account when calculating the total product concentration. Carry out extraction as described in 6.2. After extraction apply appropriate clean up procedures such as SPE. Appropriate quality controls must be included e.g. recovery assessment and correction for matrix effects made if necessary.

   b. packed in brine (and other non-edible sauces):
   Separate the mussel meat from the liquid. Rinse the mussel tissue with water, allow the mussels to drain. Weigh the drained mussel. Reconstitute the mussel tissue 50/50 tissue/water with deionised water. Homogenise the tissue and water together. Carry out extraction as described in 6.2.
   Example. For 100g of steamed mussel add 100g water.
   Formula: $weight\ water = weight\ shellfish \times \frac{50}{50}$

2. Cooked steamed mussels

Weigh the mussel tissue. Reconstitute the mussel tissue in 70/30 tissue/water with deionised water. Homogenise the tissue and water together. Carry out extraction as described in 6.2.
Example. For 100g of steamed mussel add 42.5g water.
Formula: $weight\ water = weight\ shellfish \times \frac{30}{70}$

3. Processed mussels (vacuum packed bags)

Where it is indicated that there is no added water or sauce all liquid that is present in the vacuum bag should be included in the sample. Where mussels are in the shell, the liquid should be saved, mussels shucked and the liquid added before homogenisation. Carry out extraction as described in 6.2.

**The above described procedures may be applied to shellfish species other than mussels, taking into account the known water losses during processing. Currently the losses of water during steaming and autoclaving for these shellfish species have not been described.*

This is not an exhaustive list of sample types. Advice should be sought through the individual NRL and agreed with the EURL if further clarification is needed on sample pre-treatment.

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