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Quantitative determination of marine lipophilic toxins in shellfish using LC-MS/MS

International validation study - final report

RIKILT Report 2011.008

H.J. van den Top, A. Gerssen and H.P. van Egmond

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Summary

Thirteen laboratories participated in an interlaboratory study to evaluate method performance characteristics of a liquid chromatography tandem mass spectrometric method (LC-MS/MS) for marine lipophilic shellfish toxins. Method performance characteristics were evaluated for the matrices mussels (*Mytilus edulis*), oysters (*Crassostrea gigas*) and cockles (*Cerastoderma edule*). The specific toxin analogues tested included okadaic acid (OA), dinophysistoxins-1 and -2 (DTX1,-2), azaspiracids-1, -2 and -3 (AZA1,-2,-3), pectenotoxin-2 (PTX2), yessotoxin (YTX) and 45-OH-yessotoxin (45-OH-YTX). The instrumental technique was developed as an alternative for the still widely applied biological methods (mouse or rat bioassay).

Validation was done according to the harmonised protocol for the design, conduct and interpretation of method-performance studies. Eight different test materials were sent as blind duplicates to the participating laboratories. Twelve laboratories returned results that were accepted to be included in the statistical evaluation. The method precision was expressed as HORRATs. For the individual toxins (except for 45-OH-YTX) HORRATs were found to be ≤ 1.8 (median HORRAT: 0.8) in all tested materials.

The recoveries for OA-, AZA- and YTX- group toxins were within the range of 80 – 108% and for PTX2 within the range of 62 – 93%. Based on the acceptable values for precision and recovery, it was concluded that the method is suitable for official control purposes to quantitatively determine OA- group toxins, AZA- group toxins, PTX2 and YTX- group toxins in shellfish.

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1 Introduction

Okadaic acid (OA), dinophysistoxins (DTXs), yessotoxins (YTXs), azaspiracids (AZAs) and pectenotoxins (PTXs) are the most predominant marine lipophilic shellfish toxins. At low concentrations some of these toxins can cause intoxications such as nausea, vomiting, abdominal cramps and diarrhoea. In the European Union (EU) permitted levels (PL) have been established. For OA, DTXs and PTXs together the total PL is set at 160 µg of OA equivalents per kg edible shellfish [European Parliament 2004]. The PL for YTXs and AZAs have been set at 1 mg of yessotoxin (YTX) equivalents per kg and at 160 µg of azaspiracid-1 (AZA1) equivalents per kg. In recent years the European Food Safety Authority (EFSA) has prepared several opinions on the safety levels for the most predominant marine biotoxins [EFSA 2009]. EFSA has established acute reference doses (ARfD) for the lipophilic toxins. To avoid exceeding the ARfD, a 400 grams portion of shellfish should not contain more than a certain amount of toxin. Based on these findings, EFSA recommends that the maximum concentration in shellfish meat should not exceed 45 µg of OA equivalents per kg and 30 µg of AZA1 equivalents per kg to protect consumers. If the EU would decide to lower the PL taking into account the EFSA opinions, this will have an impact for the methods that can be applied for the analysis of these toxins. For YTXs and PTXs EFSA recommends that the maximum concentration in shellfish meat should not exceed 3.75 mg YTX equivalents/kg and 120 µg PTX2 equivalents.

Furthermore, regulatory developments in the EU are directed towards replacement of rodent assays for marine lipophilic toxins by LC-MS/MS methodology as a reference method in the coming years, at the latest by the end of 2014 [European Parliament 2011].

Within the EU 6th framework project 'BIOTOX: Development of cost-effective tools for risk management and traceability systems for marine biotoxins in seafood' (contract no: 514074) an LC-MS/MS method has been developed and in-house validated at RIKILT Institute of Food Safety, the Netherlands by A. Gerssen [4, 5]. This method, as described in the Standard Operating Procedure (SOP, see annex 2), was the basis for an interlaboratory validation study, carried out in 2010, coordinated by RIKILT.

The purpose of this interlaboratory validation study was to determine accuracy, repeatability and between-laboratory reproducibility of the method as described in the SOP. The study involved the quantitative determination of free (before hydrolysis) and total (after hydrolysis) okadaic acid-group toxins [OA, dinophysistoxin-1 (DTX1) and dinophysistoxin-2 (DTX2)], azaspiracid-group toxins [AZA1, azaspiracid-2 (AZA2) and azaspiracid-3 (AZA3)], PTX2 and yessotoxin-group toxins [YTX and 45hydroxy-yessotoxin (45-OH-YTX)].

The study was organized and carried out in accordance with the Collaborative Study Guidelines of AOAC INTERNATIONAL [AOAC 2002]. Twenty laboratories in the EU were approached with an invitation to participate in the study, twelve laboratories responded positively to this invitation. One laboratory from the United States of America (USA) requested to take part in this study and this was welcomed. A list of participants is presented in table 1. This list includes the EU-RL for marine biotoxins (Vigo, ES) and 5 NRL's.

Table 1 Participants in the RIKILT interlaboratory validation study.

Laboratory	Country
Centre d'Economie Rurale (CER)	Belgium
Scientific Institute of Public Health (IPH) (NRL)	Belgium
Institut français de recherche pour l'exploitation de la mer (IFREMER)	France
Bundesinstitut für Risikobewertung (BfR) (NRL)	Germany
Chemical and Veterinary Analytical Institute Muensterland-Emscher-Lippe	Germany
Marine Institute (NRL)	Ireland
Centro Ricerche Marine (NRL)	Italy
Norwegian School of Veterinary Science (NVH)	Norway
Institut de Recerca i Tecnologia Agroalimentàries (IRTA)	Spain
Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) (EU-RL)	Spain
Centre for Environment, Fisheries & Aquaculture Science (CEFAS)	United Kingdom
Agri-Food and Biosciences Institute (AFBI) (NRL)	United Kingdom
National Oceanic and Atmospheric Administration (NOAA)	USA

2 Practice samples

Because for some participants the chromatography under alkaline conditions was relatively new, practice samples were sent to get familiar with the retention behaviour of these toxins under alkaline conditions. Participants were provided with a prepared matrix (mussel) matched standard (MMS) series and 5 methanolic extracts containing known and unknown concentrations of the toxins included in this method.

Results from these practice samples showed that participants (in most cases) obtained the required limit of detection (LOD) and limit of quantitation (LOQ) (LOQ<0.25*PL) and showed control over the method.

3 Materials

For the interlaboratory validation study eight materials were included; 5 mussel materials (*Mytilus edulis*), two oyster materials (*Crassostrea gigas*) and one cockle material (*Cerastoderma edule*). The mussel materials were prepared by blending blank tissue with highly contaminated tissue of the same matrix to achieve the target toxin level. For the oyster materials and cockle material it was not possible to obtain non-pre-processed (steam cooked) naturally contaminated material. Therefore these matrices were prepared by blending blank oyster or cockle homogenate with contaminated mussel homogenate. The percentage oyster and cockle matrix were kept as high as possible, 75%, 87% and 50% respectively for the two oyster materials and the cockle material. All materials were included in the study as blind duplicates.

A blank mussel and a blank oyster material were also included in the test sample set. One of the blank oyster materials was marked as "blank oyster" and participants were requested to prepare from this matrix a methanolic extract and spike it at 0.5 × PL. This test was intended as a check for possible different matrix effects for oyster compared to the mussel extract that participants used for preparation of the MMS.

For recovery estimations, one (mussel) homogenate was spiked using OA, AZA1 and YTX certified calibrants, at levels of 100 µg/kg for OA, 48 µg/kg for AZA1 and 300 µg/kg for YTX. A second homogenate (mussel) was spiked also with OA and AZA1 at the same levels. For method performance and recovery estimations of PTX2 participants were provided with solutions in HPLC vials (100 µl each). These solutions were used as a spiking solution (vials A, B, C, D and "recovery") to be added to three mussel and two oyster materials (see table 2). This approach was used for PTX2 as within fresh shellfish matrix PTX2 is converted rapidly to PTX2sa.

Participants received in total 23 test samples in plastic containers, each containing 1.5 – 2.0 g of shellfish homogenate.
Homogeneity tests were performed at RIKILT (according to ISO 13528:2005) on the prepared materials and materials showed to be sufficiently homogenous (RSDR(%) of 10 subsamples < 0.3*PRSDR (%)).
<u>The materials provided to the participants were:</u>

- Blind duplicates of 8 different shellfish homogenates (16)
- Blind duplicates of blank mussel (2)
- Blind duplicates of blank oyster (2)
- Duplicate mussel sample spiked for recovery estimation (2)
- Pre-release Freeze Dried Mussel Tissue material (FDMT) from NRC Canada (1)
- Pre-release calibrant from NRC DTX1 (1 ampoule)
- Pre-release calibrant from NRC DTX2 (1 ampoule)
- Pre-release calibrant from NRC AZA2 (2 ampoules)
- Pre-release calibrant from NRC AZA3 (2 ampoules)

Participants were requested to purchase the commercial available NRC certified calibrants for quantification of the other toxins (OA, AZA1, PTX2 and YTX) and to purchase also one NRC-CRM-MUS-b for analysis. All samples were individually (random) numbered per laboratory; no relation between samples could be suspected within-lab or between-lab from the numbers.

The sample key for each laboratory is given in table 2.

Table 2 Sample key for all participants.

Material	Lab 1	Lab 2	Lab 3	Lab 5	Lab 6	Lab 7	Lab 8
1	1-75420-A	2-481-A	3-4412-A	5-9332-A	6-3539-A	7-2483-A	8-4167-A
1	1-97431-A	2-8275-A	3-2799-A	5-3254-A	6-9177-A	7-8873-A	8-5921-A
2	1-37474-A	2-4465-A	3-8060-A	5-4647-A	6-3411-A	7-8049-A	8-334-A
2 spike PTX 100 µg/kg (A)	1-4008-A	2-4572-A	3-3086-A	5-8587-A	6-1766-A	7-9011-A	8-8178-A
3	1-61029-A	2-6410-A	3-1399-A	5-1728-A	6-1251-A	7-1307-A	8-5367-A
3	1-67014-A	2-268-A	3-6179-A	5-1102-A	6-9410-A	7-4300-A	8-2534-A
4	1-63885-A	2-8560-A	3-3607-A	5-9624-A	6-3470-A	7-1000-A	8-6011-A
4	1-65211-A	2-6252-A	3-664-A	5-6180-A	6-3440-A	7-8716-A	8-6929-A
5	1-57364-B	2-5003-B	3-3916-B	5-851-B	6-1202-B	7-988-B	8-7599-B
5	1-43491-B	2-7670-B	3-3931-B	5-1026-B	6-2965-B	7-8483-B	8-3651-B
6	1-96981-B	2-8687-B	3-4513-B	5-3479-B	6-4643-B	7-9385-B	8-8939-B
6	1-15451-B	2-695-B	3-5802-B	5-8405-B	6-158-B	7-3744-B	8-5377-B
7	1-68840-B	2-6731-B	3-5261-B	5-9142-B	6-8308-B	7-1771-B	8-6999-B
7	1-19870-B	2-5819-B	3-6544-B	5-7537-B	6-2604-B	7-4079-B	8-8269-B
8 spike PTX 200 µg/kg (C)	1-91342-B	2-4738-B	3-8411-B	5-8729-B	6-7007-B	7-9843-B	8-3631-B
8 spike PTX 200 µg/kg (D)	1-52040-B	2-6985-B	3-6316-B	5-4158-B	6-9597-B	7-4889-B	8-4219-B
Blank mussel	1-29548-B	2-9965-B	3-4554-B	5-8757-B	6-1356-B	7-1740-B	8-6738-B
Blank mussel	1-38714-B	2-3080-B	3-5103-B	5-3339-B	6-4270-B	7-5349-B	8-542-B
Mussel spike OA/AZA1/YTX	1-73494-B	2-9768-B	3-9530-B	5-3620-B	6-1060-B	7-9564-B	8-4386-B
Mussel spike OA/AZA1 (recovery)	Recovery	Recovery	Recovery	Recovery	Recovery	Recovery	Recovery
Blank oyster	1-1966-A	2-4936-A	3-5686-A	5-4030-A	6-8333-A	7-662-A	8-2773-A
Blank oyster	Blank Oyster	Blank Oyster	Blank Oyster	Blank Oyster	Blank Oyster	Blank Oyster	Blank Oyster
Blank mussel (B)	1-62973-A	2-3860-A	3-2797-A	5-9358-A	6-9469-A	7-6843-A	8-7777-A

Table 2 (continued) Sample key for all participants.

Material	Lab 11	Lab 14	Lab 15	Lab 17	Lab 19	Lab 20	Lab 21
1	11-1962-A	14-3978-A	15-1191-A	17-1915-A	19-1390-A	20-6633-A	4-1473-A
1	11-2965-A	14-8585-A	15-5692-A	17-7984-A	19-9236-A	20-8862-A	4-914-A
2	11-3269-A	14-1497-A	15-1417-A	17-8793-A	19-468-A	20-5978-A	4-8818-A
2 spike PTX 100 µg/kg (A)	11-5537-A	14-437-A	15-5714-A	17-2631-A	19-5863-A	20-7216-A	4-7269-A
3	11-3018-A	14-1553-A	15-6830-A	17-3149-A	19-2894-A	20-587-A	4-8368-A
3	11-6303-A	14-2159-A	15-8565-A	17-4629-A	19-9560-A	20-418-A	4-991-A
4	11-7581-A	14-5500-A	15-3992-A	17-4342-A	19-7640-A	20-5255-A	4-8269-A
4	11-1826-A	14-4599-A	15-9652-A	17-2674-A	19-8463-A	20-6447-A	4-8310-A
5	11-757-B	14-7852-B	15-2655-B	17-1425-B	19-5641-B	20-8096-B	4-9522-B
5	11-5022-B	14-8384-B	15-8659-B	17-4035-B	19-7691-B	20-2285-B	4-2107-B
6	11-3662-B	14-5404-B	15-4594-B	17-1787-B	19-5235-B	20-3572-B	4-7924-B
6	11-6578-B	14-4171-B	15-7534-B	17-8750-B	19-1682-B	20-9683-B	4-2879-B
7	11-1225-B	14-3079-B	15-7943-B	17-9833-B	19-1259-B	20-6750-B	4-2936-B
7	11-6309-B	14-756-B	15-5902-B	17-6226-B	19-4908-B	20-8413-B	4-9911-B
8 spike PTX 200 µg/kg (C)	11-8754-B	14-3803-B	15-8496-B	17-1618-B	19-753-B	20-926-B	4-4428-B
8 spike PTX 200 µg/kg (D)	11-9965-B	14-2930-B	15-8505-B	17-6827-B	19-4312-B	20-9917-B	4-5999-B
Blank mussel	11-9187-B	14-3910-B	15-7047-B	17-5896-B	19-2241-B	20-7818-B	4-5119-B
Blank mussel	11-762-B	14-3331-B	15-3623-B	17-3459-B	19-7203-B	20-1146-B	4-7614-B
Mussel spike OA/AZA1/YTX	11-4512-B	14-9067-B	15-9003-B	17-5108-B	19-1037-B	20-5742-B	4-9342-B
Mussel spike OA/AZA1 (recovery)	Recovery	Recovery	Recovery	Recovery	Recovery	Recovery	Recovery
Blank oyster	11-2946-A	14-2511-A	15-9586-A	17-5107-A	19-187-A	20-135-A	4-6821-A
Blank oyster	Blank Oyster	Blank Oyster	Blank Oyster	Blank Oyster	Blank Oyster	Blank Oyster	Blank Oyster
Blank mussel (B)	11-7475-A	14-2521-A	15-2289-A	17-9304-A	19-6245-A	20-3898-A	4-5253-A

4 Organization of the study

Description and instructions for the study

Participants received specific instructions for this study which were described in the study protocol (annex 1).

All test materials had a unique number and also had an A or a B on the label. Analyzes could be carried out in one series but when participants wished to split the series over two days this was possible. If the participant decided to split the series over two days, they were instructed to do the following:

On day one the test materials marked with "A" were analysed. Also in this series the spiked samples using the vials A and B (see table 2) were included as well as the "Blank oyster" material (see annex 1 section 3.1 for instructions about the "Blank oyster" material).

On day two the test materials marked with "B" were analysed and also in this series the spiked samples using the vials C and D (see table 2) were included. Furthermore the "Recovery sample" with the accompanying vial for this sample (see section 3.1 for instructions about the "recovery sample" material) were analysed.

The CRM-DSP-MUS-B material had to be included (one analysis) and diluted. This dilution was done in order to have a concentration of the OA group toxins in the CRM-DSP-MUS-B within the MMS working range. The dilution was done with the same blank methanolic mussel extract that was also used for preparing the MMS series. One participant indicated that they diluted the CRM-DSP-MUS-B material using methanol instead of the blank mussel extract. When statistical analysis was performed this lab showed to be an outlier. If a participant decided to split the series in two days only on one occasion the CRM-DSP-MUS-B was analysed.

The FDMT material had to be included in duplicate and analysed within one series (participants could choose on which day when they decided to split the series over two days). Three participants did not analyze the FDMT material in duplicate but only once.

Instructions for the FDMT material

Reconstitute the pre-release freeze-dried Reference Material (NRC RM-FDMT)

- After equilibration to room temperature weigh a 0.35 g portion into a centrifuge tube and record the weight accurately.
- Add 1.65 ml (or 1.65 g) of deionised water.
- Cap the centrifuge tube and vortex mix for 30 seconds to reconstitute the freeze-dried material with the water.
- Sonicate the reconstituted material for 1 minute in an ultrasonic bath.
- Extract the reconstituted material, adapting the volumes of the extraction procedure from the SOP (6.6.2) to the test portion weight of 2.0 grams instead of 1.0 grams (use: 3 x 6 ml methanol and adjust to a total volume of 20.0 ml using methanol).
- Analyse the sample extract as a normal tissue sample extract.

For the preparation of the MMS series the participants had to use a blank mussel material available from their own laboratory as this blank material was not included with the materials provided. When participants had no blank mussel available, they requested the coordinator to provide them with a blank mussel material. Two laboratories (lab 1 and lab 20) requested a blank mussel material.

All samples were analysed both unhydrolysed and hydrolysed in order to determine the total amount of OA, DTX1 and DTX2 (including acetylated ester forms).

Results were obtained by singular injection of the methanolic extract.

For uniform reporting of the results an Excel reporting sheet was designed by the coordinator. For integration of chromatograms, participants were allowed to use a smoothing factor on the chromatograms to reduce noise. Furthermore, the minimum amount of data points over a peak had to be at least 8.

For the study participants received:

Sent by courier: on the 24th of August 2010 a total of 23 test materials, vials for spiking and pre-release calibrants on dry ice.

Sent by email: on the 25th of August 2010 the SOP, the protocol for the validation study, the "arrival form" and the reporting sheet that included the unique number of the test materials to be received.

On arrival participants were requested to inspect the package on the correctness of the content and if the test materials were still frozen and the provided ampoules were intact. Furthermore they were requested to fill in the sample arrival form and return it as soon as possible to the study coordinator.

All participants indicated that the test materials were received in good condition. Two labs reported that an ampoule was broken and requested a replacement.

The QC criteria that had to be fulfilled to have "valid data" reported are summarized below:

- Drift in sensitivity (SOP paragraph 7.2.2): the slope difference for MMS series before and after the samples shall not be more than 25%.
- Each individual MMS series shall have a correlation coefficient ≥ 0.98.
- The signal-to-noise ratio of the lowest intension transition for OA, AZA1, PTX2 and YTX in the MMS 0.25 PL shall be > 6.
- Maximum deviation in the individual retention times within a series shall be ≤ 5%. "A series" is understood to be the MMS - samples - MMS sequence.

The deadline for submission of results was set to 1st October 2010. Last results were received on 15th October 2010

5 Results

5.1 Initial review of data

Results were initially reviewed and only valid data were included in the statistical evaluation. Data were considered to be "not-valid" when the criteria as mentioned in the SOP were not met:

- The calibration curve did not fulfil the correlation coefficient criteria ($R \geq 0.98$).
- Drift in the response was above 25%, the slope difference of the calibration curve of the MMS analysed before and after the samples.

Outliers

Statistical evaluation of the results was carried out following the approach described in the Collaborative Study Guidelines of AOAC INTERNATIONAL [AOAC 2002]. The use of blind duplicates facilitated the use of the Cochran test to identify laboratories showing significant greater variability among replicates (within day) when compared to the other participants (1-tail test at a probability value of 2.5%).

The Grubbs test identifies laboratories with extreme averages, the single value test (2-tail, P = 2.5%) followed by a paired value test (P = 2.5%) were performed.

Precision

The repeatability standard deviation (s_r) was calculated as

$$S_r = \sqrt{\left(\frac{\sum d_i^{\,2}}{2L}\right)}$$

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

The reproducibility standard deviation (s_R) was calculated as

$$S_R = \sqrt{\left(\frac{S_d^{\,2} + S_r^{\,2}}{n_i}\right)}$$

where

$$S_d^{\,2} = \frac{\sum \left(T_i - T_{avg}\right)^2}{n_i\left(L-1\right)}$$

being T_i the sum of the individual values for the pair in laboratory i, T_{avg} the mean of the T_i across all the laboratories or pairs, L the number of pairs and n_i the "effective" amount of replicates per laboratory. In case all laboratories performed duplicate analysis $n_i=2$.

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

$RSD_r(\%) = \frac{S_r}{\bar{x}} \times 100\%$ and $RSD_R(\%) = \frac{S_R}{\bar{x}} \times 100\%$

HorRat

HorRat value is the ratio of the RSDR(%) to the predicted (P)RSDR (%).The ratio was calculated as $HorRat = \frac{RSD_R}{PRSD_R}$

$$PRSD_R = 2^{(1-0.5 LogC)} \left(\approx 2C^{-0.1505}\right)$$

where C is the estimated mean concentration expressed as a decimal fraction. In this study, the decimal fraction for each material was calculated as: C = (mean µg toxin/kg) multiplied by 10^{-9}.

5.2 Validation study results

The method has been evaluated for the determination of OA-group toxins, AZAs, YTX, 45OH-YTX and PTX2 in three different matrices of shellfish: mussels, oysters and cockles. A total of 8 test materials were analysed in one or two days, depending on the preference of the laboratory to split the test materials into two series (both series included Quality Assurance samples). All the test materials were provided as blind duplicates. The 13 participating laboratories submitted the results for the 8 test materials. One laboratory had problems and the reporting sheets showed misidentification of toxins and missing samples. This lab adapted the method to such an extent that there was a considerable alteration of the method. Therefore, all results from this lab were considered to be "not-valid" and were excluded for further statistical evaluation.

Individual pre-release calibrants for DTX1, DTX2, AZA2 and AZA3 were made available by the NRC for this study. This makes it possible to quantify all individual toxins against the corresponding individual toxin calibrant (e.g. DTX1 using DTX1 calibrant). Furthermore, a comparison between quantifying against the individual calibrant and against the corresponding calibrant (e.g. to quantify DTX1 to the OA calibrant, as this is common practise because of the lack of individual calibrants) was incorporated in the evaluation of the study results.

The data of the method performance characteristics are provided in the tables 3 to 27.

Data on the recoveries found in the FDMT material are provided in table 28.

Additionally, the method performance characteristics were established for the total toxicity of the OA-group toxins and the AZA-group toxins both quantified against the corresponding calibrant, respectively OA and AZA1, and against the individual calibrants available and using the toxicity equivalency factors (TEFs) as published by EFSA (EFSA 2009). For YTX-group toxins the method performance characteristics were established for the total toxicity against YTX. For calculations of the total toxicity method performance characteristics, the following approach was taken for

statistical analysis. Only valid results from the individual toxins were taken into account for the total toxicity calculations. When for (one of) the individual toxins the results were considered as "not-valid" (= calibration curve slope difference > 25% and/or R < 0.980), the laboratory result for total toxicity was considered to be "not-valid" and not included in the method performance evaluation. When a laboratory had a valid result but was an "outlier" for one of the individual toxins (Cochran or Grubbs test) the result was taken into account for the method performance evaluation of the total toxicity.

Results (mean values) reported in tables 3 to 27 have NOT been corrected for recovery.

OA –group toxins results

General: "-" : no data to perform statistical calculations

Table 3 Gerssen et al. method performance characteristics for OA toxin determination against OA calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	47.8	11(0)	-	8.3	20.1	0.79
Material 2 Mussel	39.3	11(0)	-	15.5	23.0	0.88
Material 3 Mussel	89.1	11(0)	-	12.1	19.9	0.86
Material 4 Oyster	120.7	11(0)	-	7.7	16.3	0.74
Material 5 Mussel	160.4	11(0)	-	3.4	27.8	1.32
Material 6 Mussel	54.4	11(0)	-	8.0	23.0	0.93
Material 7 Cockle	297.7	11(0)	-	6.2	15.5	0.81
Material 8 Oyster	69.1	11(0)	-	8.4	19.9	0.83
FDMT material	257.6	11(0)	94	4.0	14.3	0.73
CRM-MUS-B	9507.1	9(1)	94	13.4	12.4	1.09
OA recovery (spiked tissue)	80.4	11(0)	80	17.8	25.4	1.09
OA recovery vial B	73.9	11(0)	81	n.a.	23.8	1.05

** a= number of labs remaining after removal of number of outliers indicated by (b)*
n.a.: not available (one sample provided, no duplicate measurement)

Table 4 Gerssen et al. method performance characteristics for DTX1 toxin determination against OA calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	29.5	10(1)	-	10.3	27.7	1.02
Material 2 Mussel	64.9	11(0)	-	3.9	49.4	2.04
Material 3 Mussel	< LOQ	-	-	-	-	-
Material 4 Oyster	< LOQ	-	-	-	-	-
Material 5 Mussel	58.5	10(1)	-	3.9	30.7	1.25
Material 6 Mussel	< LOQ	-	-	-	-	-
Material 7 Cockle	85.4	10(1)	-	6.1	31.1	1.34
Material 8 Oyster	13.5	10(1)	-	11.2	28.8	0.94
FDMT material	115.1	11(0)	87	5.9	31.3	1.41
CRM-MUS-B	1534.6	9(1)	118	23.4	39.2	2.61

** a= number of labs remaining after removal of number of outliers indicated by (b)*

Table 5 Gerssen et al. method performance characteristics for DTX2 toxin determination against OA calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	133.1	11(0)	-	6.3	13.4	0.62
Material 2 Mussel	< LOQ	-	-	-	-	-
Material 3 Mussel	75.6	11(0)	-	13.7	21.5	0.91
Material 4 Oyster	172.9	11(0)	-	4.6	21.0	1.01
Material 5 Mussel	< LOQ	-	-	-	-	-
Material 6 Mussel	14.2	11(0)	-	26.0	32.1	1.06
Material 7 Cockle	171.6	11(0)	-	6.3	16.7	0.80
Material 8 Oyster	103.9	10(1)	-	4.9	20.7	0.92
FDMT material [1)]	765.1	11(0)	129	4.7	13.6	0.82

** a= number of labs remaining after removal of number of outliers indicated by (b)*
[1)] mean value (µg/kg) > two times the maximum level in MMS series

Table 6 Gerssen et al. method performance characteristics for DTX1 toxin determination against DTX1 calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	36.8	11(0)	-	10.9	16.7	0.63
Material 2 Mussel	69.6	11(0)	-	5.6	12.7	0.53
Material 3 Mussel	< LOQ	-	-	-	-	-
Material 4 Oyster	< LOQ	-	-	-	-	-
Material 5 Mussel	70.7	9(1)	-	4.0	12.0	0.50
Material 6 Mussel	< LOQ	-	-	-	-	-
Material 7 Cockle	106.5	10(0)	-	7.2	13.7	0.61
Material 8 Oyster	17.3	10(0)	-	12.3	26.1	0.88
FDMT material	125.2	10(0)	94	4.1	18.2	0.83
CRM-MUS-B	1380.5	9(0)	106	13.7	28.1	1.84

** a= number of labs remaining after removal of number of outliers indicated by (b)*

Table 7 Gerssen et al. method performance characteristics for DTX2 toxin determination against DTX2 calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	111.0	11(0)	-	5.9	17.2	0.77
Material 2 Mussel	< LOQ	-	-	-	-	-
Material 3 Mussel	63.5	11(0)	-	11.6	18.8	0.78
Material 4 Oyster	142.0	11(0)	-	4.5	16.7	0.78
Material 5 Mussel	< LOQ	-	-	-	-	-
Material 6 Mussel	13.5	10(0)	-	29.2	36.4	1.19
Material 7 Cockle	144.1	10(0)	-	5.0	14.0	0.65
Material 8 Oyster	87.7	10(0)	-	4.5	15.7	0.68
FDMT material [1)]	643.4	11(0)	108	5.0	21.7	1.27

** a= number of labs remaining after removal of number of outliers indicated by (b)*
[1)] mean value (µg/kg) > two times the maximum level in MMS series

Table 8 Gerssen et al. method performance characteristics for OA-Group total toxicity (sum of OA, DTX1 and DTX2 expressed as OA equivalents) determination against OA calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	161.4	11(0)	-	5.7	16.7	0.79
Material 2 Mussel	108.9	11(0)	-	7.9	27.9	1.25
Material 3 Mussel	136.0	11(0)	-	11.1	19.3	0.89
Material 4 Oyster	225.7	11(0)	-	5.1	15.8	0.79
Material 5 Mussel	211.5	10(1)	-	2.3	22.1	1.09
Material 6 Mussel	64.7	11(0)	-	9.6	23.0	0.95
Material 7 Cockle	499.0	11(0)	-	5.7	18.8	1.06
Material 8 Oyster	143.8	10(1)	-	4.6	17.2	0.80
FDMT material	831.8	11(0)	109	3.7	10.6	0.64

** a= number of labs remaining after removal of number of outliers indicated by (b)*

Table 9 Gerssen et al. method performance characteristics for OA-Group total toxicity (sum of OA, DTX1 and DTX2 expressed as OA equivalents) determination against individual calibrants (OA, DTX1 and DTX2).

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	151.1	11(0)	-	5.8	14.2	0.67
Material 2 Mussel	115.4	10(0)	-	6.9	13.7	0.62
Material 3 Mussel	128.4	11(0)	-	11.0	18.8	0.86
Material 4 Oyster	206.9	11(0)	-	5.3	14.7	0.72
Material 5 Mussel	242.4	10(0)	-	2.2	21.9	1.10
Material 6 Mussel	65.2	10(0)	-	10.4	16.6	0.69
Material 7 Cockle	483.4	10(0)	-	3.3	13.0	0.73
Material 8 Oyster	137.0	10(0)	-	5.0	15.9	0.74
FDMT material	777.7	10(0)	102	3.8	16.8	1.01

** a= number of labs remaining after removal of number of outliers indicated by (b)*

OA –group toxins HYDROLYSED results

Table 10 Gerssen et al. method performance characteristics for OA toxin determination after hydrolysis against OA calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	137.8	9(0)	-	5.7	23.9	1.11
Material 2 Mussel	66.0	9(0)	-	5.6	24.7	1.02
Material 3 Mussel	177.6	9(0)	-	12.9	24.9	1.20
Material 4 Oyster	249.1	9(0)	-	5.1	27.5	1.40
Material 5 Mussel [1)]	618.3	9(0)	-	7.3	31.1	1.81
Material 6 Mussel	74.2	9(0)	-	10.1	23.5	0.99
Material 7 Cockle [1)]	976.9	9(0)	-	29.9	29.2	1.82
Material 8 Oyster	243.3	9(0)	-	6.1	22.8	1.15
FDMT material [1)]	671.8	10(0)	n.a.	2.6	21.5	1.27

** a= number of labs remaining after removal of number of outliers indicated by (b)*
[1)] mean value (µg/kg) > two times the maximum level in MMS series

Table 11 Gerssen et al. method performance characteristics for DTX1 toxin determination after hydrolysis against OA calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	36.4	9(0)	-	9.4	20.4	0.77
Material 2 Mussel	66.6	9(0)	-	7.8	22.1	0.92
Material 3 Mussel	< LOQ	-	-	-	-	-
Material 4 Oyster	< LOQ	-	-	-	-	-
Material 5 Mussel	97.7	8(1)	-	5.9	27.8	1.22
Material 6 Mussel	-	-	-	-	-	-
Material 7 Cockle	132.5	8(1)	-	20.2	34.5	1.59
Material 8 Oyster	23.9	8(1)	-	11.4	24.5	0.87
FDMT material	110.3	10(0)	n.a.	5.0	38.1	1.71

** a= number of labs remaining after removal of number of outliers indicated by (b)*

Table 12: Gerssen et al. method performance characteristics for DTX2 toxin determination after hydrolysis against OA calibrant

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	189.6	9(0)	-	6.3	36.6	1.78
Material 2 Mussel	< LOQ	-	-	-	-	-
Material 3 Mussel	107.1	9(0)	-	18.4	35.2	1.57
Material 4 Oyster	260.5	9(0)	-	7.3	40.1	2.05
Material 5 Mussel	< LOQ	-	-	-	-	-
Material 6 Mussel	15.7	9(0)	-	19.2	45.6	1.52
Material 7 Cockle	261.6	9(0)	-	30.5	29.7	1.52
Material 8 Oyster	196.2	9(0)	-	8.2	42.3	2.07
FDMT material [1)]	935.8	9(1)	n.a.	2.5	28.7	1.78

** a= number of labs remaining after removal of number of outliers indicated by (b)*
[1)] mean value (µg/kg) > two times the maximum level in MMS series

Table 13 Gerssen et al. method performance characteristics for DTX1 toxin determination after hydrolysis against DTX1 calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	55.3	11(0)	-	12.3	24.6	1.00
Material 2 Mussel	98.2	11(0)	-	9.7	23.7	1.04
Material 3 Mussel	< LOQ	-	-	-	-	-
Material 4 Oyster	< LOQ	-	-	-	-	-
Material 5 Mussel	137.9	10(0)	-	7.0	19.4	0.90
Material 6 Mussel	< LOQ	-	-	-	-	-
Material 7 Cockle	188.4	9(1)	-	5.6	17.8	0.87
Material 8 Oyster	33.7	10(0)	-	11.1	21.4	0.80
FDMT material	143.6	9(0)	n.a.	5.4	19.6	0.91

** a= number of labs remaining after removal of number of outliers indicated by (b)*

Table 14 Gerssen et al. method performance characteristics for DTX2 toxin determination after hydrolysis against DTX2 calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	182.1	10(0)	-	7.1	20.3	0.98
Material 2 Mussel [1)]	9.4	10(0)	-	18.1	65.9	2.04
Material 3 Mussel	105.6	10(0)	-	15.8	27.3	1.21
Material 4 Oyster	251.5	10(0)	-	7.1	27.9	1.42
Material 5 Mussel	< LOQ	-	-	-	-	-
Material 6 Mussel	13.5	11(0)	-	41.1	49.4	1.62
Material 7 Cockle	233.3	11(0)	-	25.1	29.4	1.47
Material 8 Oyster	160.0	11(0)	-	11.9	27.2	1.29
FDMT material [2)]	933.9	11(1)	n.a.	5.1	17.3	1.07

** a= number of labs remaining after removal of number of outliers indicated by (b)*
[1)] mean value is at the average LOQ as reported by participants (see table 29)
[2)] mean value (µg/kg) > two times the maximum level in MMS series

Table 15 Gerssen et al. method performance characteristics for OA-Group total toxicity determination after hydrolysis against OA calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	287.9	9(0)	-	4.5	22.2	1.15
Material 2 Mussel	138.7	9(0)	-	5.1	17.1	0.80
Material 3 Mussel	244.2	9(0)	-	14.1	22.7	1.15
Material 4 Oyster	407.8	9(0)	-	4.9	28.2	1.54
Material 5 Mussel	745.6	9(0)	-	6.5	32.4	1.94
Material 6 Mussel	89.2	9(0)	-	8.1	23.0	0.99
Material 7 Cockle	1304.3	9(0)	-	27.7	30.5	1.98
Material 8 Oyster	390.4	9(0)	-	5.5	28.1	1.52

** a= number of labs remaining after removal of number of outliers indicated by (b)*

Table 16 Gerssen et al. method performance characteristics for OA-Group total toxicity determination after hydrolysis against individual calibrants (OA, DTX1 and DTX2).

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	299.0	9(0)	-	4.0	22.0	1.15
Material 2 Mussel	168.4	9(0)	-	5.6	24.0	1.15
Material 3 Mussel	243.4	9(0)	-	13.6	25.1	1.27
Material 4 Oyster	401.3	9(0)	-	4.4	27.5	1.50
Material 5 Mussel	757.2	9(0)	-	6.5	28.4	1.70
Material 6 Mussel	84.9	9(0)	-	8.6	23.4	1.01
Material 7 Cockle	1293.4	9(0)	-	28.8	27.5	1.79
Material 8 Oyster	371.4	9(0)	-	5.2	22.8	1.23

** a= number of labs remaining after removal of number of outliers indicated by (b)*

AZA –group toxins results

Table 17 Gerssen et al. method performance characteristics for AZA1 toxin determination against AZA1 calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	71.0	11(0)	-	11.2	17.3	0.73
Material 2 Mussel	< LOQ	-	-	-	-	-
Material 3 Mussel	115.5	11(0)	-	2.8	9.1	0.41
Material 4 Oyster	194.5	11(0)	-	2.9	12.2	0.60
Material 5 Mussel	< LOQ	-	-	-	-	-
Material 6 Mussel	252.2	10(0)	-	3.3	10.2	0.52
Material 7 Cockle	142.4	10(0)	-	2.7	11.4	0.53
Material 8 Oyster	< LOQ	-	-	-	-	-
FDMT material [1)]	615.4	10(0)	86	3.1	12.2	0.71
AZA1 recovery (spiked tissue)	39.4	10(0)	82	8.7	20.8	0.80

** a= number of labs remaining after removal of number of outliers indicated by (b)*
[1)] mean value (µg/kg) > two times the maximum level in MMS series

Table 18 Gerssen et al. method performance characteristics for AZA2 toxin determination against AZA1 calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	16.0	9(2)	-	3.7	11.2	0.37
Material 2 Mussel	< LOQ	-	-	-	-	-
Material 3 Mussel	24.8	10(1)	-	2.9	10.3	0.37
Material 4 Oyster	40.4	10(1)	-	2.7	12.3	0.48
Material 5 Mussel	< LOQ	10(1)	-	-	-	-
Material 6 Mussel	56.6	10(0)	-	3.3	14.4	0.58
Material 7 Cockle	32.1	10(0)	-	3.7	13.0	0.48
Material 8 Oyster	< LOQ	-	-	-	-	-
FDMT material	150.9	10(0)	76	3.2	17.2	0.81

** a= number of labs remaining after removal of number of outliers indicated by (b)*

Table 19 Gerssen et al. method performance characteristics for AZA3 toxin determination against AZA1 calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	37.3	11(0)	-	5.0	19.7	0.75
Material 2 Mussel	3.8	11(0)	-	11.6	50.7	1.37
Material 3 Mussel	55.9	10(1)	-	2.2	18.4	0.74
Material 4 Oyster	54.5	10(1)	-	3.7	21.0	0.85
Material 5 Mussel	< LOQ	-	-	-	-	-
Material 6 Mussel	126.7	9(1)	-	2.1	15.4	0.71
Material 7 Cockle	72.6	9(1)	-	3.3	17.0	0.72
Material 8 Oyster	-	-	-	-	-	-
FDMT material	113.4	9(1)	61	1.3	18.2	0.82

** a= number of labs remaining after removal of number of outliers indicated by (b)*

Table 20 Gerssen et al. method performance characteristics for AZA2 toxin determination against AZA2 calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	17.2	12(0)	-	10.6	20.0	0.68
Material 2 Mussel	< LOQ	-	-	-	-	-
Material 3 Mussel	27.5	12(0)	-	3.0	9.5	0.35
Material 4 Oyster	44.6	12(0)	-	5.6	8.8	0.34
Material 5 Mussel	< LOQ	-	-	-	-	-
Material 6 Mussel	59.8	12(0)	-	8.3	9.1	0.37
Material 7 Cockle	35.5	11(1)	-	5.7	7.2	0.27
Material 8 Oyster	< LOQ	-	-	-	-	-
FDMT material	165.0	12(0)	83	2.9	8.9	0.42

** a= number of labs remaining after removal of number of outliers indicated by (b)*

Table 21 Gerssen et al. method performance characteristics for AZA3 toxin determination against AZA3 calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	53.5	11(1)	-	3.7	6.3	0.25
Material 2 Mussel	5.0	12(0)	-	14.4	50.3	1.41
Material 3 Mussel	84.4	11(1)	-	3.4	5.7	0.25
Material 4 Oyster	81.4	11(1)	-	3.9	6.5	0.28
Material 5 Mussel	< LOQ	-	-	-	-	-
Material 6 Mussel	186.0	12(0)	-	1.9	10.1	0.49
Material 7 Cockle	108.9	11(1)	-	3.5	7.1	0.32
Material 8 Oyster	< LOQ	-	-	-	-	-
FDMT material	171.9	12(0)	92	2.9	14.7	0.70

** a= number of labs remaining after removal of number of outliers indicated by (b)*

Table 22 Gerssen et al. method performance characteristics for AZA-Group total toxicity determination against AZA1 calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	151.9	11(0)	-	4.6	15.1	0.71
Material 2 Mussel	11.1	11(0)	-	6.6	69.1	2.19
Material 3 Mussel	247.2	11(0)	-	1.9	15.8	0.80
Material 4 Oyster	352.9	11(0)	-	3.2	15.3	0.82
Material 5 Mussel	< LOQ	-	-	-	-	-
Material 6 Mussel	547.9	10(0)	-	2.9	15.1	0.86
Material 7 Cockle	311.4	10(0)	-	2.3	15.4	0.81
Material 8 Oyster	< LOQ	-	-	-	-	-
FDMT material	1053.9	10(0)	79	2.7	13.2	0.83

** a= number of labs remaining after removal of number of outliers indicated by (b)*

Table 23 Gerssen et al. method performance characteristics for AZA-Group total toxicity determination against individual calibrants (AZA1, AZA2 and AZA3).

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	175.0	11(0)	-	4.4	12.3	0.59
Material 2 Mussel	13.1	11(0)	-	8.0	65.8	2.14
Material 3 Mussel	280.1	11(0)	-	1.9	9.0	0.47
Material 4 Oyster	385.2	11(0)	-	3.0	10.2	0.55
Material 5 Mussel	< LOQ	-	-	-	-	-
Material 6 Mussel	615.2	10(0)	-	2.5	8.9	0.52
Material 7 Cockle	350.8	10(0)	-	2.3	10.9	0.58
Material 8 Oyster	< LOQ	-	-	-	-	-
FDMT material	1146.5	10(0)	86	2.6	10.4	0.66

** a= number of labs remaining after removal of number of outliers indicated by (b)*

PTX2 results

Table 24 Gerssen et al. method performance characteristics for PTX2 toxin determination against PTX2 calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	< LOQ	-	-	-	-	-
Material 2 Mussel spiked 100 µg/kg	82.3	10(0)	85	n.a.	10.2	0.47
Material 3 Mussel	< LOQ	-	-	-	-	-
Material 4 Oyster	< LOQ	-	-	-	-	-
Material 5 Mussel	9.7	11(0)	-	10.9	34.0	1.06
Material 6 Mussel	< LOQ	-	-	-	-	-
Material 7 Cockle	15.2	9(2)	-	7.4	9.7	0.32
Material 8 Oyster spiked 200 µg/kg	186.3	11(0)	92	4.3	20.6	1.00
FDMT material	73.5	11(0)	62	4.5	26.1	1.10
recovery sample Mussel spiked at 100 µg/kg	74.6	11(0)	79	9.8	17.2	0.73

** a= number of labs remaining after removal of number of outliers indicated by (b)*
n.a.: not available (no duplicate measurements)

YTX - group results

Table 25 Gerssen et al. method performance characteristics for YTX toxin determination against YTX calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	143.9	9(1)	-	12.5	12.5	0.58
Material 2 Mussel	1023.2	9(1)	-	5.4	12.8	0.81
Material 3 Mussel	713.2	9(1)	-	6.3	8.3	0.49
Material 4 Oyster	< LOQ	-	-	-	-	-
Material 5 Mussel	290.6	11(0)	-	11.1	16.8	0.87
Material 6 Mussel	206.9	10(1)	-	12.3	13.5	0.66
Material 7 Cockle	172.7	9(2)	-	12.4	9.9	0.48
Material 8 Oyster 1)	39.1	11(0)	-	6.2	48.4	1.86
FDMT material	371.2	11(1)	83	14.2	18.3	0.99
YTX recovery (spiked tissue)	267.1	11(0)	89		31.2	1.60

** a= number of labs remaining after removal of number of outliers indicated by (b)*
mean value is below the average LOQ as reported by participants (see table 29)

Table 26 Gerssen et al. method performance characteristics for 45-OH-YTX toxin determination against YTX calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	98.6	10(0)	-	8.0	32.5	1.43
Material 2 Mussel	651.6	10(0)	-	3.4	40.0	2.35
Material 3 Mussel	418.2	9(1)	-	7.2	27.3	1.49
Material 4 Oyster	< LOQ	-	-	-	-	-
Material 5 Mussel	177.7	11(0)	-	15.3	33.9	1.63
Material 6 Mussel	114.9	11(0)	-	18.1	37.2	1.68
Material 7 Cockle	108.6	11(0)	-	19.7	40.4	1.81
Material 8 Oyster	< LOQ	-	-	-	-	-

** a= number of labs remaining after removal of number of outliers indicated by (b)*

Table 27 Gerssen et al. method performance characteristics for YTX-group total toxicity determination against YTX calibrant.

	mean value (µg/kg)	No. of labs a(b)*	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
Material 1 Mussel	254.7	9(0)	-	6.3	24.7	1.26
Material 2 Mussel	1702.2	9(0)	-	3.5	25.5	1.73
Material 3 Mussel	1109.9	8(1)	-	6.4	8.0	0.51
Material 4 Oyster	< LOQ	-	-	-	-	-
Material 5 Mussel	462.2	10(0)	-	12.3	21.8	1.21
Material 6 Mussel	310.4	9(1)	-	11.1	16.4	0.86
Material 7 Cockle	280.8	8(2)	-	15.7	14.7	0.76
Material 8 Oyster	< LOQ	-	-	-	-	-

** a= number of labs remaining after removal of number of outliers indicated by (b)*

Table 28 Recovery estimations for the FDMT material.

FDMT	recovery in %	
	vs OA	vs individual toxins
OA	94	n.a.
DTX1	87	94
DTX2	129	108
	vs AZA1	vs individual toxins
AZA1	86	n.a.
AZA2	76	83
AZA3	61	92
PTX2	-	62
YTX	-	83

Table 29 Average LOD / LOQ values reported by participants for the different toxins (n=12).

	Method LOD [Gerssen 2009] (µg/kg)	Average LOD (µg/kg)	Average LOQ (µg/kg)
OA	9.1	2.7 ± 2.1	8.7 ± 10.0
DTX1	n.a.	4.8 ± 4.6	11.8 ± 10.6
DTX2	n.a.	3.1 ± 3.0	9.1 ± 9.9
AZA1	1.1	1.6 ± 1.5	4.4 ± 3.1
AZA2	n.a.	1.5 ± 1.5	4.2 ± 3.0
AZA3	n.a.	2.6 ± 2.6	7.1 ± 4.8
PTX2	7.4	1.8 ± 1.6	4.9 ± 3.5
YTX	2.2	16.2 ± 24.3	52.7 ± 73.7

n.a.: not available

*Table 30 Oyster matrix spiked at 0.5*PL quantified against MMS in mussel matrix.*

	mean value (µg/kg)	# labs with valid data*	target value in µg/kg	Recovery (%)	RSDr (%)	RSDR (%)	HorRat
OA oyster spike at 0.5 PL	75.2	8	80.0	94.0	3.3	19.5	0.83
DTX1 oyster spike at 0.5 PL	76.5	8	80.0	95.6	17.3	14.2	0.60
DTX2 oyster spike at 0.5 PL	73.1	8	80.0	91.4	4.9	15.0	0.63
AZA1 oyster spike at 0.5 PL	71.1	8	80.0	88.9	2.4	28.7	1.21
AZA2 oyster spike at 0.5 PL	80.8	8	80.0	101.1	1.0	12.3	0.53
AZA3 oyster spike at 0.5 PL	79.0	9	80.0	98.7	1.9	14.2	0.61
PTX2 oyster spike at 0.5 PL	81.7	8	80.0	102.1	6.8	21.7	0.93
YTX oyster spike at 0.5 PL	486.1	8	500.0	97.2	4.9	6.4	0.36

** No outlier data evaluation performed, all reported values are taken into account*

6 Discussion and acknowledgements

The quantification of an individual toxin against the corresponding toxin (e.g. DTX1 against OA) showed a ratio RSDr:RSDR that lies outside the expected ratio of 2:3 or 1:2, in fact for material 2 the RSDR is up to > 10 x higher then the RSDr. The between laboratory variance in table 4 (material 2) is (much) larger then the within laboratory variance. When however for this same material the DTX1 individual toxin is quantified against the DTX1 calibrant, the RSDr : RSDR ratio drops near to the expected ratio of 1:2.

The assumption that the response in the mass spectrometer of OA is equal to the response of DTX1 and thus DTX1 can be quantified expecting the same RSDR either against OA or DTX1 may not be true since there can be some variation between labs. This is not a shortcoming of the method but is a consequence of the common approach when the individual calibrant is not available.

When comparing the results of DTX1 quantified against OA with DTX1 quantified against DTX1 (table 4 and table 6) it shows that for all materials the DTX1 content is estimated to be 1.2 x higher when quantified against DTX1 then as quantified against OA (n=6, c.v. 7.5%). A similar effect is observed for the results of DTX1 after hydrolysis (table 11 and table 13) where the DTX1 content is estimated to be 1.4 x higher when quantified against DTX1 then as quantified against OA (n=6, c.v. 5.1%). The same is the case for AZA3 (table 19 and table 21), in these tables the same pattern is shown for the RSDr : RSDR ratio. When comparing the AZA3 content quantified against AZA3, with AZA3 quantified against AZA1, it shows that the AZA3 content quantified against AZA3 is about 1.5 x higher (n=7, c.v. 4.7%). For DTX2 and AZA2 the effect is less noticeable.

The average reported LOQs and LODs by participants are given in table 29. The LOQ for this method as reported by participants in this study is low and makes this method suitable for the determination of these marine lipophilic toxins when the EU-regulatory limits would be lowered for OA- and AZA-group toxins as a consequence of the EFSA opinions [EFSA 2009].

Table 30 shows the values participants obtained by spiking an oyster extract at the level of 0.5* PL for each of the toxins tested. This table shows that the toxin content in an oyster matrix which was quantified against the MMS prepared in mussel extract is very close to the target value. Therefore it can be concluded that using an MMS prepared in mussel extract is an effective approach to correct for matrix effects originating from an oyster shellfish species. This is in line with the results obtained in the RIKILT in-house validation of this method where the matrix effects of oysters, cockles, ensis and mussels were compared for the different toxins and no significant differences were observed [Gerssen 2010].

We would like to express a big "thank you" to all of the participants for their good work!
We would like to thank especially:
The EU-RL on Marine Biotoxins (Vigo, Spain), because of their continuous support in the preparation and organization of this study.

The NRC-Canada for making several (pre-release) reference materials available for this study.

A research paper about this validation study will be published in 2011 [van den Top 2011]

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Annex I Validation study protocol

Validation study of the method:

Quantitative determination of marine lipophilic toxins in shellfish using LC-MS/MS

Study instructions

24 August 2010

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THIS IS A STUDY OF THE METHOD, NOT OF THE LABORATORY.
THE METHOD MUST BE FOLLOWED AS CLOSELY AS PRACTICABLE, AND ANY DEVIATIONS FROM THE METHOD AS DESCRIBED, NO MATTER HOW TRIVIAL THEY MAY SEEM, MUST BE NOTED ON THE REPORT FORM.

IN CASE OF DOUBT THE PARTICIPANT SHALL CONTACT THE COORDINATOR BEFORE PROCEEDING WITH THE ANALYSIS.

1. Aim of the interlaboratory validation study

The purpose of this validation study is to determine precision (both repeatability and between-laboratory reproducibility) and bias of the method "Liquid chromatography–tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions" (Gerssen et al, 2009).
The study involves the quantitative determination of the free and total Okadaic acid group toxins (OA, DTX1 and DTX2), the Pectenotoxin group toxins (PTX1 and PTX2), the Azaspiracid group toxins (AZA1, AZA2 and AZA3) and the Yessotoxin group toxins (YTX, homo-YTX, 45-OH-YTX and 45-OH-homo-YTX). These are the lipophilic marine shellfish toxins, currently regulated in the EU.

2. Test materials

Materials including different species of shellfish (mussels, oysters and cockles) naturally contaminated at various levels and blanks will be tested. All materials are included as blind duplicates.
The test materials were prepared by combining naturally contaminated shellfish tissues with blank tissues at various proportions to obtain the desired toxin level. The materials were prepared by combining tissues from the same shellfish species if possible. In cases where this was not possible, the proportion of contaminated material from one species with blank material from a different species was kept as small as possible.

Participants will receive a total of 23 test samples (each of about 1.5 – 2.0 grams), a pre-release reference material (Freeze-dried mussel tissue – FDMT of about 1 gram freeze-dried material), 5 vials containing a spiking solution and 6 ampoules containing pre-release calibrant solutions (approx 0.5 ml per ampoule) for the following toxins:

- 1 ampoule of DTX1 at concentration: 18.7 µmol/l (=15.3 µg/ml)
- 1 ampoule of DTX2 at concentration: 9.7 µmol/l (= 7.8 µg/ml)
- 2 ampoules of AZA2 at concentration: 1.5 µmol/l (= 1.28 µg/ml)
- 2 ampoules of AZA3 at concentration: 1.4 µmol/l (= 1.16 µg/ml)

For the study 1 ampoule per toxin is needed.

Homogeneity of the test materials has been verified by LC-MS analysis according to IUPAC and ISO guidelines. Stability will be verified during the period of study.

All test materials must arrive frozen and must be stored upon arrival in the freezer (≤ -15 °C). Let test materials thaw only just before proceeding to analysis.

Mix contents of test sample containers well before weighing the test portion for analysis.

3. Experimental design

All samples have a unique number and sample numbers also have an A or a B on the label. Analyses can be carried out in one series. When participants wish to split the series over 2 days this is possible.

If participants want to split the series over two days, they shall do the following:

Day one:
Analyze ONLY the test materials marked with "A" and include in this series the spiked samples using the vials A and B (see reporting sheet); include also the "Blank oyster" material (see section 3.1 for instructions about the "Blank oyster" material).

Day two:
Analyze ONLY the test materials marked with "B" and include in this series the spiked samples using the vials C and D (see reporting sheet); include also the "Recovery sample" with the accompanying vial for this sample (see section 3.1 for instructions about the "recovery sample" material).

The CRM DSP MUS-B material shall be included (one analysis) and diluted to obtain a value that is within the MMS working range using the blank mussel extract also used for preparing the MMS series. Participants can choose on which day they perform this analysis when they decide to split the series over two days.

The FDMT material must be included in DUPLICATE and analyzed within one series (participants can choose on which day when they decide to split the series over two days).

Instructions for the FDMT material

Reconstitute the pre-release freeze-dried Reference Material (NRC RM-FDMT)

- After equilibration to room temperature weigh 0.35 g portion into a centrifuge tube and record the weight accurately.
- Add 1.65 ml (or 1.65 g) of deionised water.
- Cap the centrifuge tube and vortex mix for 30 seconds to reconstitute the freeze-dried material with the water.
- Sonicate the reconstituted material for 1 minute in an ultrasonic bath.
- Extract the reconstituted material, adapting the volumes of the extraction procedure from the SOP (6.6.2) to the test portion weight of 2.0 grams instead of 1.0 grams (use: 3 x 6 ml methanol and adjust to total volume of 20.0 ml using methanol).
- Analyse the sample extract as a normal tissue sample extract.

A blank mussel material for the preparation of the MMS series is not included in the materials provided for the study. Participants shall use a blank mussel material available in their own laboratory for the preparation of the MMS series. When participants have no blank mussel available, they can send an email request to the coordinator to provide them with a blank mussel material.

Analyse ALL samples both unhydrolysed and hydrolysed.

Follow the order of analysis as mentioned in the SOP section 6.6.4.

Injections on LC-MS/MS: singular injections.

Reporting of results: For integration of chromatograms, participants can use a smoothing factor on the chromatograms to reduce noise, however the minimum amount of data points over a peak shall be at least 8.

Please use the reporting sheet provided for reporting of results.
When the sample series is divided over two days participants shall provide one individual reporting sheet for each day of analysis.

3.1 Recovery instructions

Participants will have received 5 HPLC vials (labelled: A; B; C; D and "recovery"). These vials contain a spiking solution that participants have to add to the assigned samples numbers that are mentioned in the reporting sheet (under "general" tab). Participants shall follow the instructions for spiking as mentioned below.

Instructions for the spiking procedure for vials A, B, C, D and "recovery":

- Weigh out 1 gram of the accompanying sample.
- Add the total content of the vial to the corresponding weighed out test portion.
- RINSE the HPLC vial twice using 200 µl pure methanol and add to the weighed out test portion.
- Extract and analyze the test portion as a normal tissue material.

Instructions for "Blank oyster" material (labeled "Blank oyster").
Participants have also received a "Blank oyster" material.
Participants shall use the extract of this oyster material to prepare one point of the calibration series and that point shall be the MMS 0.5 PL level.

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4. Method

The method to be used for this study is described in the SOP:
Quantitative determination of marine lipophilic toxins in shellfish using LC-MS/MS, final version (date: 24-08-2010)

Please adhere to the method as close as possible. The fragments for the different toxins mentioned in the SOP (table 7 on page 12) are examples. If participants have the experience that other transitions perform better, they can adapt and report the transitions used in the reporting sheet.

The QC criteria that have to be fulfilled are summarized below:

- Drift in sensitivity (part 7.2.2 page 15): the slope difference for MMS series before and after the samples shall not be more than 25 %.
- Each individual MMS series shall have a correlation coefficient ≥0.98.
- The signal-to-noise ratio of the lowest intension transition for OA, AZA1, PTX2 and YTX in the MMS 0.25 PL shall be > 6.
- Maximum deviation in the individual retention times within a series shall be ≤ 5 %. "A series" is understood to be the MMS - samples - MMS sequence.

The reporting sheet will also calculate the ion ratio and indicate if the ion ratio is within EU criteria for residue analysis. This ion ratio calculation will be used for information only in this study and will not be used as a QC criterion.

The limit of detection (LOD) and the limit of quantification (LOQ) will be determined by each participant for the individual toxins and recorded in the reporting sheet.

5. Statistical analysis of results and study report

Initial evaluation of the data will be done according to IUPAC/AOAC guidelines. ONLY valid data will be included in the statistical analysis. Valid data are values for which the participant and the study director have no reason to suspect as being wrong. Invalid data will result from the method protocol not being followed on the key points (extraction, LC conditions and the use of the prescribed MMS) or from a situation, where the QC criteria are not fulfilled.

6. Participants

Thirteen laboratories from nine European countries and one laboratory from the USA will participate in this validation study.

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7. Timetable

The timetable for the validation study is summarised in the table below.

Task	Deadline
Sending of materials to participants	24 August 2010
Submission of results by participants to RIKILT	01 October 2010
Preliminary evaluation of results	October 2010
Report on the validation study	January 2011

8. Reference documents

- Arjen Gerssen, Patrick P.J. Mulder, Mairead A. McElhinney, Jacob de Boer; "Liquid chromatography–tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions"; *Journal of Chromatography A*, 1216 (2009) 1421–1430

- AOAC Guidelines for collaborative study procedures to validate characteristics of a method of analysis (appendix D). 2002. AOAC International

Annex II SOP Validation study

Final SOP validation study | Date: August 24, 2010

SOP

Validation study

Quantitative determination of marine lipophilic toxins in shellfish using LC-MS/MS

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Appendix 1: Proposed structures for the MS/MS fragments

Appendix 2: Chromatogram

Appendix 3: Excel result sheet

Appendix 4: Checklist SOP determination marine lipophilic toxins in shellfish

2

1 INTRODUCTION AND OBJECTIVES

Okadaic acid, dinophysistoxins, yessotoxins, azaspiracids and pectenotoxins are the most predominant lipophilic marine shellfish toxins. At low concentrations some of these toxins can cause intoxications such as nausea, vomiting, abdominal cramps and diarrhoea. In the European Union permitted levels have been established: for okadaic acid, dinophysistoxins and pectenotoxins the total permitted level is set at 160 µg/kg shellfish. The permitted levels (PLs) for yessotoxins and azaspiracids have been set at 1000 µg/kg and at 160 µg/kg, respectively [1]. Current regulatory developments in the EU are directed towards replacement of rodent assays for marine lipophilic toxins by LC-MS/MS methodology in shellfish by 2012. This SOP describes the quantitative determination of these lipophilic toxins in shellfish by using LC-MS/MS. The SOP is the basis for an interlaboratory validation study, carried out in 2010, and coordinated by RIKILT Institute of Food Safety, the Netherlands

2 ABBREVIATIONS

45OH YTX	45 hydroxy Yessotoxin
45OH Homo YTX	45 hydroxy Homo Yessotoxin
AZA1	Azaspiracid-1
AZA2	Azaspiracid-2
AZA3	Azaspiracid-3
DTX1	Dinophysistoxin-1
DTX2	Dinophysistoxin-2
DTX3	Dinophysistoxin-3 (the generic term for a wide variety of OA, DTX1 and DTX2 toxins containing fatty acid chains).
Homo YTX	Homo Yessotoxin
MLT	Marine lipophilic toxin
MMS	Matrix matched standards
MRM	Multiple reaction monitoring
OA	Okadaic acid
PL	Permitted level
PTX1	Pectenotoxin-1
PTX2	Pectenotoxin-2
SPE	Solid phase extraction
SRM	Single reaction monitoring
YTX	Yessotoxin

3 PRINCIPLE

MLTs are extracted from shellfish homogenate by mixing 1 gram of homogenate with 3 ml methanol. After centrifugation the supernatant is transferred to a volumetric flask of 10 ml. The extraction step is repeated twice. After the third extraction the volumetric flask is made up with methanol till the mark. The extract is filtered and transferred to a vial. This methanolic extract is analyzed by LC-MS/MS for the presence of MLTs. Additionally, a hydrolysis step is incorporated to determine the total amount of DTX3. DTX3 is the generic term for a wide variety of OA, DTX1 and DTX2 toxins containing fatty acid chains. The fatty acid chains are removed by hydrolysis of the methanolic extract with sodium hydroxide at a temperature of 76°C during 45 minutes. After 45 minutes the extract is cooled to room temperature and neutralized with hydrochloric acid. The hydrolysed extract is filtered and analysed. The increase in peak area for OA, DTX1 and DTX2 after hydrolysis (as compared to before hydrolysis) is a measure for the amount of DTX3 present.

4 REAGENTS

All chemicals should at least be of pro-analysis quality or higher. With "water" we mean water purified by a Milli-Q purification system with a minimal resistance of 10 MΩ/cm. References to products or producers are just for general information and do not imply that other products or producers with the same or similar characteristics are ruled out.

4.1 Standards

4.1.1 Okadaic acid (OA) (NRC-CNRC Institute for Marine Biosciences, Canada)
4.1.2 Yessotoxin (YTX) (NRC-CNRC Institute for Marine Biosciences, Canada)
4.1.3 Azaspiracid-1 (AZA1) (NRC-CNRC Institute for Marine Biosciences, Canada)
4.1.4 Pectenotoxin-2 (PTX2) (NRC-CNRC Institute for Marine Biosciences, Canada)
4.1.5 Dinophysistoxin-1 (DTX1) (NRC-CNRC Institute for Marine Biosciences, **Pre-release**)
4.1.6 Dinophysistoxin-2 (DTX2) (NRC-CNRC Institute for Marine Biosciences, **Pre-release**)
4.1.7 Azaspiracid-2 (AZA2) (NRC-CNRC Institute for Marine Biosciences, **Pre-release**)
4.1.8 Azaspiracid-3 (AZA3) (NRC-CNRC Institute for Marine Biosciences, **Pre-release**)

4.2 Chemicals

4.2.1 Acetonitrile (e.g. Biosolve 01203502)
4.2.2 Methanol (e.g. Biosolve 136835)
4.2.3 Sodium hydroxide (e.g. Merck 106498)
4.2.4 Hydrochloric acid 32% (e.g. VWR 20256393)
4.2.5 Ammonia 25% (e.g. Merck 105432)

4.3 Reagents

4.3.1 Mobile phase A: 0.05 v/v % ammonia in water (pH 11)

Add with a positive displacement pipette 0.5 ml ammonia (4.2.5) to 1000 ml water and mix. This solution is stored at room temperature and can be used for 1 month.

4.3.2 Mobile phase B: 0.05 v/v % ammonia in 90% acetonitrile
Add with the help of graduated cylinders 900 ml acetonitrile (4.2.1) and 100 ml water together in a bottle of 1000 ml. Add with a positive displacement pipette 0.5 ml ammonia (4.2.5) and mix. This solution is stored at room temperature and can be used for 1 month.

4.3.3 Sodium hydroxide 2.5 mol/L
Dissolve 10.0 g sodium hydroxide (4.2.3) in 90 ml water in a volumetric flask of 100 ml and make up to the mark with water. This solution is stored at room temperature and can be used for 1 month.

4.3.4 Hydrochloric acid 2.5 mol/L
Add 24 ml hydrochloric acid (4.2.4) to a volumetric flask of 100 ml and make up to the mark with methanol (4.2.2). This solution is stored at room temperature and can be used for 1 month.

4.4 Standard solutions

4.4.1 Primary standard solutions MLTs
The ampoules containing the various toxins (4.1.1 to 4.1.4) are supplied with a certified concentration that varies per toxin. The ampoules 4.1.5 to 4.1.8 are for use in this validation study only and are not certified. In table 1 example calculations are shown for the dilution factors of the standards. Check the concentration of new ampoules as the concentration may change. A certain volume from the ampoules is diluted with methanol (4.2.2) to the amount and concentration of the primary standard solution as described in table 1, dilutions are made on a volume base. Store the primary standard solutions at -20°C. Under these circumstances the solutions can be stored for 12 months.

Table 1: Concentrations and dilution factors of the various toxins (Attention please: concentrations in new ampoules can deviate)

Toxin	Concentration (µg/ml)	Dilution factor for primary standard	Concentration primary standard solution (µg/ml))	Required amount of primary solvent (µl) for preparation of 4.4.2 and 6.5.2
OA	14.3	1.43	10	250
PTX2	8.6	2.15	4	250
YTX	5.3	1.06	5	1000
DTX1 (provided for study)	15.3	1.53	10	250
DTX2 (provided for study)	7.8	1.56	5	500
AZA1	1.24	1.24	1	500
AZA2 (provided for study)	1.28	1.28	1	500
AZA3 (provided for study)	1.16	1.16	1	500

4.4.2 Mixed standard solution

Pipette the amounts of primary standard solutions (4.4.1) as described in table 2 in a 4 ml vial (5.7), and add an additional amount of methanol (4.2.2) as indicated in the table and mix. Total volume of the AZA mix is 1.25 ml and total volume of the OA/DTX/PTX/YTX mix is 1.6 ml. Store the mixed standard solution at -20°C. Under these circumstances the solution can be stored for 6 months.

Table 2: Pipette scheme mixed standard solution

Toxin	Concentration primary standard solution (µg/ml) (4.4.1)	Amount to pipette in the 4 ml vial (µl)	Add methanol	Concentration in the mixed standard solution (ng/ml)
OA	10	64	384	400
PTX2	4	160		400
YTX	5	800		2500
DTX1	10	64		400
DTX2	5	128		400
Pipette a separate mixed standard solution for the AZA toxins.				
AZA1	1	400	50	320
AZA2	1	400		320
AZA3	1	400		320

4.4.3 Standard solution OA, DTX1 and DTX2 (OA-group) for hydrolysis (320 ng/ml for each individual toxin)

Pipette 1744 µl methanol (4.2.2) in a 4 ml vial (5.7), add 64 µl primary standard solution OA (4.4.1) as described in table 2, add 64 µl primary standard solution DTX1, and 128 µl primary

standard solution DTX2 and mix. Store the standard solution at -20°C. Under these circumstances the solution can be stored for 6 months.

5 APPARATUS

5.1 Analytical balance with a minimal weigh range of 0 until 100 gram with a precision of 0.1 mg (e.g. Mettler/Toledo AT261 Deltarange)

5.2 Balance with a minimal weigh range of 0 until 1500 gram with a precision of 0.1 g (e.g. Mettler/Toledo PB1502)

5.3 Centrifuge suitable for 12 ml centrifuge tubes (5.6) (e.g. MSE Falcon 6/300 suitable for 2000 *g*)

5.4 Ultra-Turrax® (e.g. Janke & Kunkel T25)

5.5 Filter disc units (e.g. Acrodisc 25 mm 0.2 µm HT Tuffryn)

5.6 Polypropylene centrifuge tubes 12 ml with screw cap (e.g. Greiner 163275)

5.7 Vial 4 ml with screw cap with silicone septum (e.g. vials Grace Alltech 98110, caps Grace Alltech 98610)

5.8 Medicine bottle 100 ml (e.g. Emergo, 280603)

5.9 Waterbath with a minimum range up to 80°C

5.10 Syringe (e.g. Terumo syringe 10 ml, SS 10ES)

5.11 Vial 1.5 ml (e.g. Grace Alltech 98239)

5.12 Aluminium vial caps, suitable for 1.5 ml vials (e.g. Grace Alltech 3112746)

5.13 Various pipettes and volumetric flasks

a. e.g. Eppendorf 1-5 ml
b. e.g. Gilson 100-1000 µl
c. e.g. Gilson Microman (M25) 2-25 µl
d. e.g. Gilson Microman (M100) 10-100 µl
e. e.g. Gilson Microman (M250) 50-250 µl
f. e.g. Gilson Microman (M1000) 100-1000 µl
g. 10 ml volumetric flasks

5.14 Small knife

5.15 Oyster glove (optional)

5.16 Plastic jar, lockable (e.g. Schotte e.g. 2594000)

5.17 Vortex (e.g. Vibrofix VF 1)

5.18 Oyster opener

5.19 Grinding machine (e.g. Moulinex, La Moulinette)

5.20 Multipulse Vortex (e.g. Glas-Col)

5.21 LC-MS/MS system existing out of:

5.21.1 Shimadzu UFLC (or comparable)

5.21.2 Analytical column suitable for alkaline conditions (pH 1 -12): e.g. Waters X-Bridge™ C_{18} 150 × 3 mm, 5 or 3.5 µm particles

5.21.3 Mass spectrometer: Micromass Quattro Ultima, equipped with an ESI interface (or comparable)

6 PROCEDURE

6.1 General

This SOP describes the quantitative method for determining MLTs in shellfish meat. In appendix 4, paragraphs 6.4 until 6.6.5 are shown in a checklist, which can be used as a tool during execution of the SOP.

6.2 Precautionary measures

Take adequate precautionary measures to prevent inhalation and skin contact with the standards, reagents and other solutions used. Wear a lab coat and use where necessary a fume hood, safety glasses and safety gloves.

6.3 Pre-treatment of the sample

Remove shellfish meat from the shell by using a small knife (5.14) and if desired an oyster glove (5.15). Place the blade between the two shells and carefully jiggle until the shellfish opens. Remove the seawater that is still clinging to it and remove the shellfish meat. Collect for each sample at least 100 g meat in a lockable plastic jar (5.16). Open oysters with a special oyster tool (5.18). Homogenize the shellfish meat with an Ultra turrax (5.4) or grinding machine (5.19).

6.4 Test portion

The amount of shellfish homogenate examined is 1.0 gram.

6.5 Preparation of matrix matched standards (MMS)

6.5.1 MMS

Choose as blank shellfish homogenate a sample, where in previous analyses no MLTs were detected. Weigh 3 individual test portions of 1.0 ± 0.05 gram of the same blank shellfish homogenate each in an individual plastic tube of 12 ml (5.6) and add 3 ml methanol (4.2.2)[1]. Vortex mix during 1 minute using the multipulse vortex (5.20). Centrifuge 5 minutes at 2000 *g*, transfer the supernatant to a volumetric flask of 10 ml (5.13). Repeat this extraction twice, after the third extraction make up to the mark with methanol. Pool the three blank extracts and mix prior to preparation of the matrix matched standards. Filter the extract through a 0.2 µm membrane filter (5.5). Pipette with a positive displacement pipette aliquots of 865 µl extract in six individual 12 ml plastic tubes (5.6). Next add the standard solution according to table 3.

[1] This is done in order to get enough blank extract for the preparation of the MMS series

Table 3: Preparation MMS

×PL	MMS (µg/kg)		Blank extract (µl)	Mixed standard solution (4.4.2) (µl)	Mixed standard solution (4.4.2) (µl)	methanol (4.2.2) (µl)
	OA, DTX, AZA and PTX	YTX		OA, DTX,PTX, and YTX mix	AZA mix	
0	0	0	865	0	0	135
0.125	20	125	865	5	6.3	123.7
0.25	40	250	865	10	12.5	112.5
0.5	80	500	865	20	25.0	90
1	160	1000	865	40	50.0	45
1.5	240	1500	865	60	75.0	0

Homogenize each spiked extract carefully during 5 seconds using a vortex (5.17). Transfer the spiked extract to a 1.5 ml HPLC vial (5.11) and directly seal the vial with an aluminium crimp cap (5.12) or use a HPLC vial with screw cap and silicone liner. For hydrolysis, make individual MMS (6.5.2).

6.5.2 MMS for hydrolysis

Pipette with a positive displacement pipette aliquots of 1.85 ml blank extract (6.5.1) in six individual 12 ml plastic tubes (5.6). Add standard solution OA/DTX1/DTX2 (OA-group) (4.4.3) as described in table 4.

Table 4: Preparation MMS for hydrolysis

×PL	MMS (µg/kg)	blank extract (µl)	Mixed standard solution OA / DTX1 and DTX2 for hydrolysis (4.4.3) (µl)	methanol (4.2.2) (µl)
	OA-group			
0	0	1850	-	150
0.125	20	1850	12.5	137.5
0.25	40	1850	25	125
0.5	80	1850	50	100
1	160	1850	100	50
1.5	240	1850	150	-

Homogenize each spiked extract carefully during 5 seconds using a vortex (5.17). Subsequently follow the procedure as described in 6.6.3.

6.6 Description of the analysis procedure

6.6.1 General

Weigh a test portion of 1.0 ± 0.05 gram (5.2) shellfish homogenate in a plastic centrifuge tube (5.6).

6.6.2 Extraction and filtration

Add to the test portion 3 ml methanol (4.2.2) and mix during 1 minute using a multipulse vortex (5.20). Centrifuge (5.3) the extract for 5 minutes at 2000 *g*. Transfer the supernatant to a volumetric flask of 10 ml (5.13). Repeat the extraction procedure twice. After the third extraction, fill the flask till the mark with methanol (4.2.2). Filter at least 3 ml of the extract through a 0.2 µm membrane filter (5.5), collect the filtered extract in a 12 ml tube (5.6). Transfer the purified extract to a 1.5 ml HPLC vial (5.11) and directly seal the vial with an aluminium crimp cap (5.12) or use a HPLC vial with screw cap and silicone liner.

6.6.3 Hydrolysis

Pipette with a positive displacement pipette 1.2 ml of the extract (6.6.2) or 1.2 ml each of the MMS extracts (6.5.2) in a 12 ml plastic tube (5.6). Add 150 µl sodium hydroxide solution (4.3.3), seal the tube tightly and homogenize. Place the tube during 45 minutes in a water bath (5.9) at 76°C. Remove after 45 minutes the tube from the water bath and cool to room temperature. Neutralize the extract by adding 150 µl hydrochloric acid solution (4.3.4). Homogenize the mixture by vortex mixing. Transfer the hydrolysed extract to a 1.5 ml HPLC vial (5.11) and seal the vial directly with an aluminium crimp cap (5.12) or use a HPLC vial with screw cap and silicone liner.

6.6.4 Order of analysis

Analyse the MMS and the sample extracts in the order as given below, if necessary the series can be split into a series with and without hydrolysis.

- MMS PL, initial test (6.7)
- methanol
- MMS (6.5.1)
- methanol
- sample extracts (6.6.2)
- methanol
- MMS (6.5.1)
- methanol
- MMS after hydrolysis (6.5.2 / 6.6.3)
- methanol
- sample extracts after hydrolysis (6.6.3)
- methanol
- MMS after hydrolysis (6.5.2 / 6.6.3)

6.6.5 LC conditions

Flow rate : 0.4 ml/min
Injection volume : 10 µl
Column temperature : 40°C

Vial tray temperature : 10 °C
Gradient : See table 5
Run time : 20 minutes
Solvent delay : 0-3 and 16-19 minutes (depending on elution times of toxins)

Table 5: overview gradient HPLC system *

Time (min)	Mobile phase A (4.3.1) (%)	Mobile phase B (4.3.2) (%)
0	90	10
1	90	10
10	10	90
13	10	90
15	90	10
19	90	10

* Gradient should be adjusted in such a way that there is at least a resolution of 1 (separation of 94% or better) between OA and DTX2.

6.6.6 MS conditions

The conditions given below are guidelines; in practice other settings may be required to obtain an optimal performance of the LC-MS/MS system.

Table 6: Guidelines MS setting on a Quattro Ultima

	Quattro Ultima
Ionization mode	ESI, negative and ESI positive
Capillary voltage	2.8 kV
Cone voltage	See table 7
Source temperature	120 °C
Desolvation temperature	350 °C
Desolvation gas flow	600 L/hr
Cone gas flow	100 L/hr
Multiplier	750
CID gas	Argon, $2.5*10^{-3}$ mbar

The precursor ions fragment to structural related product ions. In table 7 the theoretical monoisotopic masses and allowed deviations of the precursor ions and corresponding product ions are shown.

Table 7: Indicative values MS/MS fragmentation conditions (monoisotopic mass ± allowed deviation, see appendix 1 for proposed fragments)

Compound	Ionisation mode	Precursor ion	Product ion*	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
Okadaic acid	ESI -	803.5 ± 0.5	255.2 ± 0.5	0.06	60	45
			113.1 ± 0.5	0.06	60	50
Dinophysistoxin-1	ESI -	817.5 ± 0.5	255.2 ± 0.5	0.06	60	45
			113.1 ± 0.5	0.06	60	50

Dinophysistoxin-2	ESI -	803.5 ± 0.5	255.2 ± 0.5	0.06	60	45
			113.1 ± 0.5	0.06	60	50
Yessotoxin	ESI -	570.4 ± 0.5	467.4 ± 0.5	0.06	75	30
			396.4 ± 0.5	0.06	75	30
Homo Yessotoxin	ESI -	577.4 ± 0.5	474.4 ± 0.5	0.06	75	30
			403.4 ± 0.5	0.06	75	30
45 Hydroxy Yessotoxin	ESI -	578.4 ± 0.5	467.4 ± 0.5	0.06	75	30
			396.4 ± 0.5	0.06	75	30
45 Hydroxy Homo Yessotoxin	ESI -	585.4 ± 0.5	474.4 ± 0.5	0.06	75	30
			403.4 ± 0.5	0.06	75	30
Azaspiracid-1	ESI +	842.5 ± 0.5	824.5 ± 0.5	0.10	35	30
			672.7 ± 0.5	0.10	35	40
Azaspiracid-2	ESI +	856.5 ± 0.5	838.5 ± 0.5	0.10	35	30
			672.7 ± 0.5	0.10	35	40
Azaspiracid-3	ESI +	828.5 ± 0.5	810.5 ± 0.5	0.10	35	30
			658.7 ± 0.5	0.10	35	40
Pectenotoxin-1	ESI +	892.5 ± 0.5	839.5 ± 0.5	0.10	40	30
			213.1 ± 0.5	0.10	40	30
Pectenotoxin-2	ESI +	876.5 ± 0.5	823.5 ± 0.5	0.10	40	30
			213.1 ± 0.5	0.10	40	30

* The product ion with the highest intensity is underlined.

6.7 Initial test of the LC-MS/MS system

Check the system performance as well as the retention times and time windows of the various toxins. For toxins for which no standard is available, a mixture of previously positive extracts can be prepared and used later on for this system check. The system should be able to detect the product ion with the lowest intensity with a s/n ratio of 6 or more for all toxins in the 0.25 PL level of the MMS.

7 RESULTS

In this chapter equations are described which are used to calculate various values in the result sheet. Additionally the criteria to judge the correctness of an analysis are described.

7.1 Equations

Equation I: Calculation of the concentration in the sample (X)

$$X = \left(\frac{area - b}{a}\right) \times toxicity\ factor$$

Where:

X	= concentration of marine lipophilic toxin (µg/kg).
area	= area of the highest intensity product ion.
b	= intercept of the calibration curve (from the linear regression curve*).
a	= slope of the calibration curve (from the linear regression curve*).
toxicity factor	= toxicity factor used to calculate concentrations in equivalents (table 8).

* Plot the area of the MMS (6.5.1) measured before and after the sample extracts as function of the added levels. Calculate the linear regression using the least squares method.

Equation II: Calculation of DTX3 concentration

$$X_{DTX3} = X_{after\ hydrolysis} - X_{before\ hydrolysis}$$

Where:

X_{DTX3} = Concentration DTX3 form of OA, DTX1 and DTX2 (µg/kg).

$X_{after\ hydrolysis}$ = Concentration of OA or DTX1 or DTX2 after hydrolysis (µg/kg).

$X_{before\ hydrolysis}$ = Concentration of OA or DTX1 or DTX2 before hydrolysis (µg/kg).

Calculate separately for OA, DTX1 and DTX2

Equation III: Calculation of the relative deviation of the retention time (ΔRT)

$$\Delta RT = \left(\frac{RT_{sample} - RT_{avg}}{RT_{avg}} \right) \times 100\%$$

Where:

ΔRT = relative deviation of the retention time of the MLT in the sample extract, compared to the MMS (6.5.1) analysed before and after the sample extracts.

RT_{sample} = retention time of the MLT in the sample extract.

RT_{avg} = average retention time of the MLT present in the fortified MMS (6.5.1) analysed before and after the sample extracts.

7.2 Performance criteria for acceptance of the series

7.2.1 General

For acceptance of the analysis result, the performance criteria (stated below) have to be met. The toxin concentrations are automatically calculated when the result sheet (appendix 3) is used. At the same time the excel sheet will show if the calculated analysis result meets the stated criteria.

7.2.2 Drift in sensitivity

The MMS analysed before the sample extracts compared with the MMS analysed after the sample extracts are indicative for the performance of the LC-MS/MS system. For both MMS series, the area of the product ions is plotted as function of the added concentration to the sample (µg/kg). Two slopes for both MMS series are constructed, which may not differ by more than 25%.

7.2.3 Linearity

The MMS (6.5.1 and 6.5.2) are used to determine the linearity of the LC-MS/MS system and to determine if the sample pre-treatment is done correctly. For both MMS series, the area of the highest intensity product ion is plotted as function of the added concentration in the sample (µg/kg). Apply linear regression using the least squares method. A correlation coefficient is calculated, which should be 0.980 or higher.

7.2.4 First line control

In the blank sample (MMS 0 µg/kg) no MLTs should be detected (or < 10% of the 1.0 PL). In the 0.25 PL MMS MLTs OA, YTX, AZA1 and PTX2 should be detected. Fill in the first line control in the result sheet.

7.2.5 Maximum deviation retention time

Determine the average retention time of the toxins in the MMS analysed before and after the sample extracts. The deviation in the individual retention times may not differ more than 5% compared to the average retention time of the toxins in the MMS.

7.2.6 Quantitation

The concentration of the marine lipophilic toxin in the sample is expressed as µg/kg and is calculated using the linear regression curves calculated from the MMS series analysed before and after the sample extracts. Calculate the concentrations using equation I and II (7.1). For the toxins were no standard is available the linear regression curve of the corresponding standard group is used (table 8). Furthermore, toxicity factors are used to calculate the total concentration. The concentration is automatically calculated using the result sheet (appendix 3).

Table 8: Toxins and analogs, of which regression curves are used in absence of relevant toxin standards, and toxicity factors for the various toxins (according to EFSA)

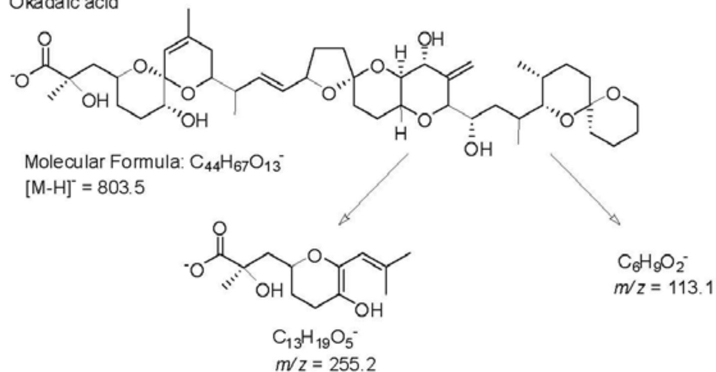
Toxin	Regression curves to be used to calculate concentration in equivalents of	Toxicity factors
OA	OA	1
DTX1	OA	1
DTX2	OA	0.6
YTX	YTX	1
Homo YTX	YTX	1
45OH YTX	YTX	1
45OH Homo YTX	YTX	0.5
AZA1	AZA1	1
AZA2	AZA1	1.8
AZA3	AZA1	1.4
PTX1	PTX2	1
PTX2	PTX2	1

8 REFERENCES

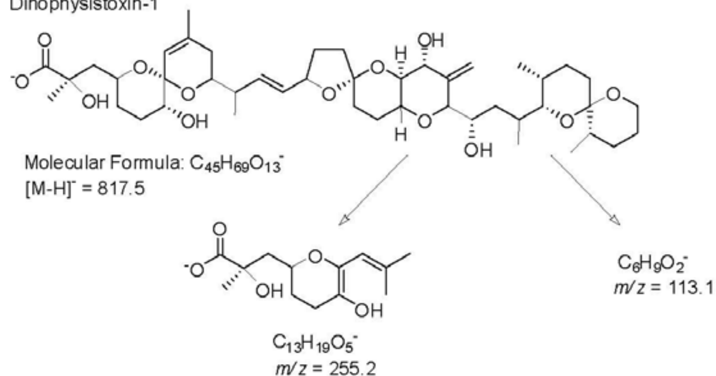
1. European Commission (2004) Commission Directive 2004/853/EC Specific hygiene rules for food of animal origin. Off J Eur Commun L226:22-82

Appendix 1 Proposed structures for the MS/MS fragments observed for the various toxins

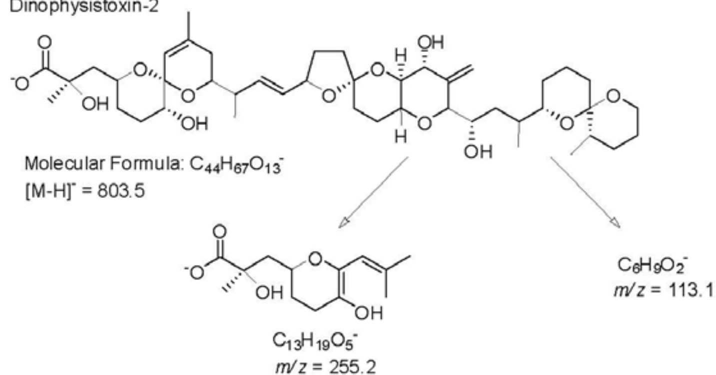
Okadaic acid

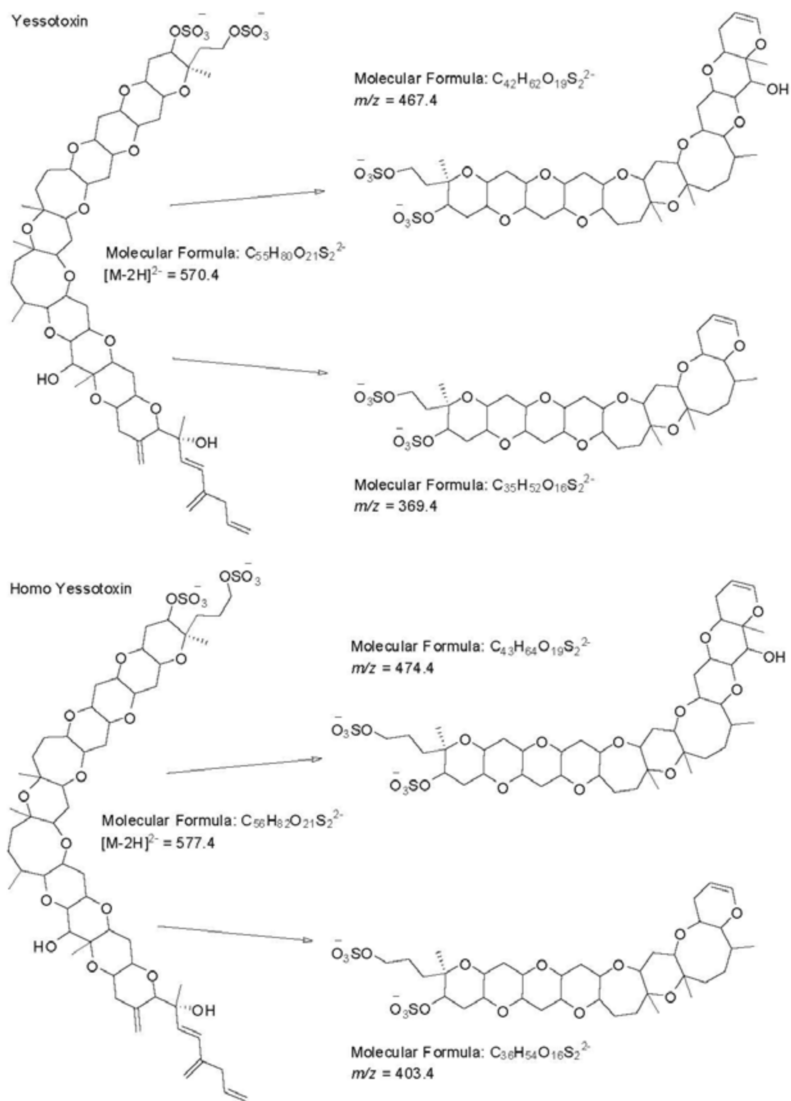


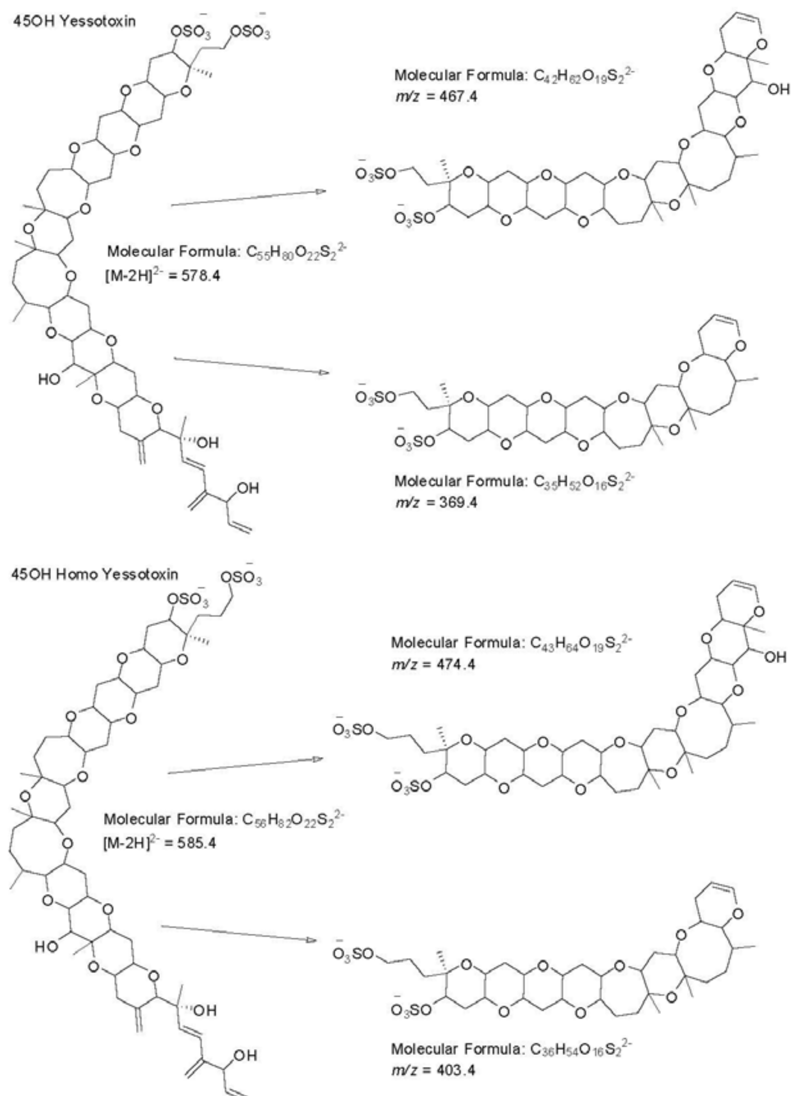
Dinophysistoxin-1

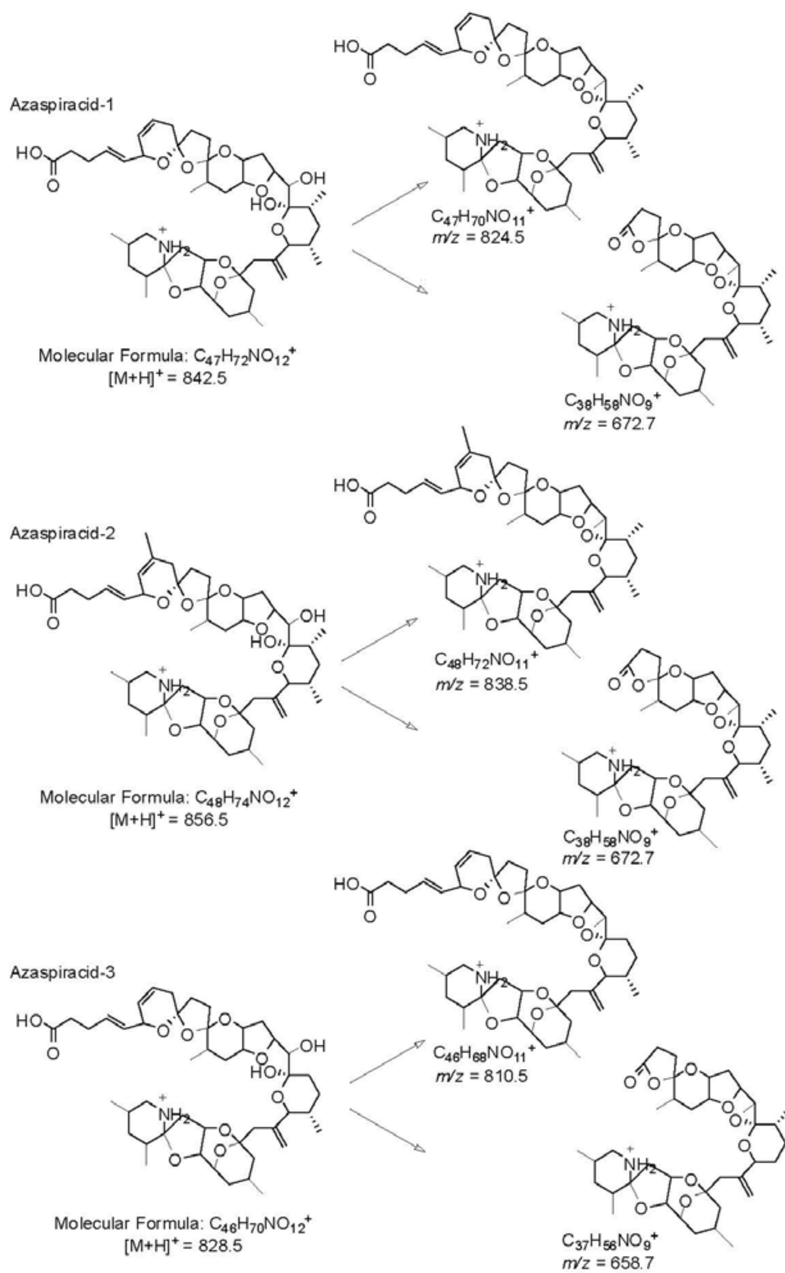


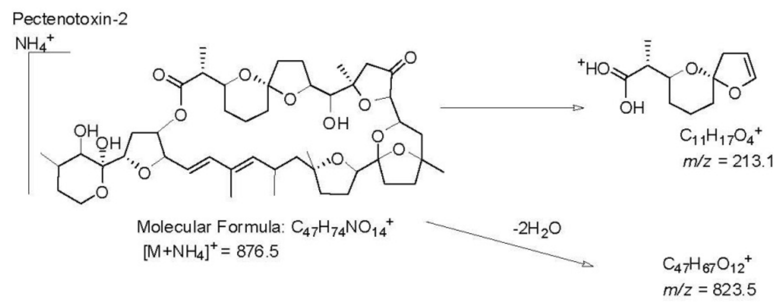
Dinophysistoxin-2



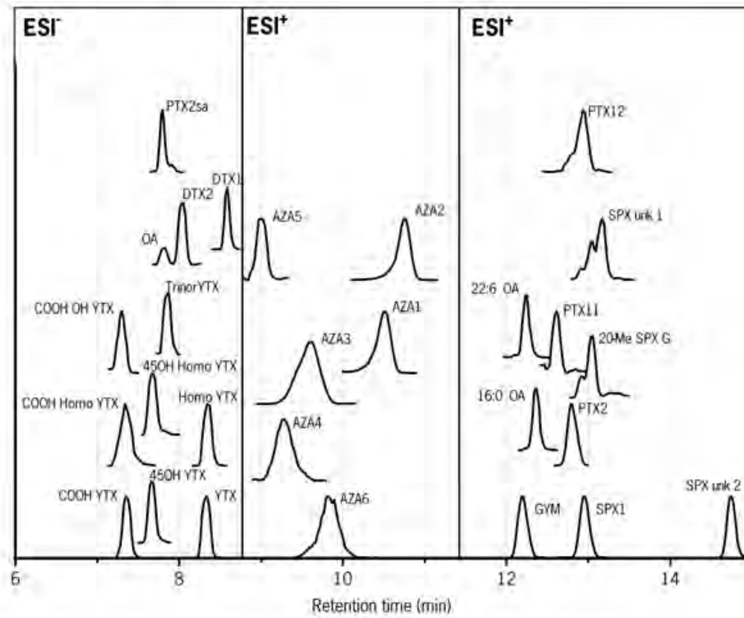

Yessotoxin
Molecular Formula: $C_{55}H_{80}O_{21}S_2^{2-}$
$[M-2H]^{2-}$ = 570.4
Molecular Formula: $C_{42}H_{62}O_{19}S_2^{2-}$
m/z = 467.4
Molecular Formula: $C_{35}H_{52}O_{16}S_2^{2-}$
m/z = 369.4
Homo Yessotoxin
Molecular Formula: $C_{56}H_{82}O_{21}S_2^{2-}$
$[M-2H]^{2-}$ = 577.4
Molecular Formula: $C_{43}H_{64}O_{19}S_2^{2-}$
m/z = 474.4
Molecular Formula: $C_{36}H_{54}O_{16}S_2^{2-}$
m/z = 403.4


45OH Yessotoxin
Molecular Formula: $C_{55}H_{80}O_{22}S_2^{2-}$
$[M-2H]^{2-}$ = 578.4
Molecular Formula: $C_{42}H_{62}O_{19}S_2^{2-}$
m/z = 467.4
Molecular Formula: $C_{35}H_{52}O_{16}S_2^{2-}$
m/z = 369.4
45OH Homo Yessotoxin
Molecular Formula: $C_{56}H_{82}O_{22}S_2^{2-}$
$[M-2H]^{2-}$ = 585.4
Molecular Formula: $C_{43}H_{64}O_{19}S_2^{2-}$
m/z = 474.4
Molecular Formula: $C_{36}H_{54}O_{16}S_2^{2-}$
m/z = 403.4


Azaspiracid-1
Molecular Formula: $C_{47}H_{72}NO_{12}^+$
$[M+H]^+ = 842.5$
$C_{47}H_{70}NO_{11}^+$
$m/z = 824.5$
$C_{38}H_{58}NO_9^+$
$m/z = 672.7$
Azaspiracid-2
Molecular Formula: $C_{48}H_{74}NO_{12}^+$
$[M+H]^+ = 856.5$
$C_{48}H_{72}NO_{11}^+$
$m/z = 838.5$
$C_{38}H_{58}NO_9^+$
$m/z = 672.7$
Azaspiracid-3
Molecular Formula: $C_{46}H_{70}NO_{12}^+$
$[M+H]^+ = 828.5$
$C_{46}H_{68}NO_{11}^+$
$m/z = 810.5$
$C_{37}H_{56}NO_9^+$
$m/z = 658.7$


Pectenotoxin-2
NH_4^+
OH
OH
OH
Molecular Formula: $C_{47}H_{74}NO_{14}^+$
$[M+NH_4]^+ = 876.5$
^+HO
OH
$C_{11}H_{17}O_4^+$
$m/z = 213.1$
$-2H_2O$
$C_{47}H_{67}O_{12}^+$
$m/z = 823.5$

Appendix 2 Chromatogram



Appendix 3 Excel result sheet

MMS

Analysed before samples

Concentration [µg/kg]	RT OA	Area 255.2	Area 113.1
0			
20			
40			
80			
160			
240			

rel. error RT [%]	ion ratio	err. ion ratio [%]

	Symbol	Value
slope	a1	
intercept	b1	
correlation	r1	

Analysed after samples

Concentration [µg/kg]	RT OA	Area 255.2	Area 113.1
0			
20			
40			
80			
160			
240			

rel. error RT [%]	ion ratio	err. ion ratio [%]

	Symbol	Value
slope	a2	
intercept	b2	
correlation	r2	

decay sensitivity: |a1-a2|/a1*100% =

Average RT

Accuracy MMS 80 µg/kg =

avg ion ratio	
stdev ion ratio	
CV% ion ratio	

	Symbol	Value
slope	a	
intercept	b	
correlation	r	

ion ratio

Acceptance criteria:

Description	Value	Criteria	Fulfil criteria
Decay sensitivity		=< 25%	
Linearity		=> 0,990	
Sensitivity MMS 0.5 PL		S/N>3	
CV ion ratio		=< 0,5*EU	
Max. err ion ratio MMS (20-240 µg/kg)		=<	
Max. rel. err. RT MMS (20-240 µg/kg)		=< 5.0%	

Series accepted:
Date:

Pagina 1

Samples

max error ion ratio conform EU-criteria =

Sample Description / file name	Sample Number	RT OA	Area 255.2	Area 113.1

ion ratio	error ion ratio [%]	error RT [%]	Concentration [µg/kg]	Result

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Appendix 4 Checklist SOP Determination marine lipophilic toxins in shellfish

Performed by:	
Date of analysis:	
Lab journal / page:	

MMS Samples

☐ ☐ Weigh 1.0 g ± 0.05 g in a plastic tube of 12 mL

☐ ☐ 1- Add 3 ml methanol and vortex mix 1 minute

☐ ☐ Centrifuge 5 minutes at 2000 *g*

☐ ☐ Transfer supernatant to volumetric flask of 10 ml

☐ ☐ 2- Add 3 ml methanol and vortex mix 1 minute

☐ ☐ Centrifuge 5 minutes at 2000 *g*

☐ ☐ Transfer supernatant to same volumetric flask of 10 ml

☐ ☐ 3- Add 3 ml methanol and vortex mix 1 minute

☐ ☐ Centrifuge 5 minutes at 2000 *g*

☐ ☐ Transfer supernatant to same volumetric flask of 10 ml and make up to mark with methanol

☐ ☐ Filter extract using 0.2 µm filter, collect filtrate in a 12 ml tube

☐ ☐ Transfer extract to vial and cap

Hydrolysis of extracts

☐ 1- MMS Pipette 6× 1.85 ml extract in individual 12 ml tubes and spike according to table 10 and mix

☐ ☐ Pipette 1.2 ml of 1-MMS and of the extracts in 12 ml tubes

☐ ☐ Add 150 µl 2.5M NaOH and mix

☐ ☐ Place extracts in water bath at 76°C during 45 minutes

☐ ☐ Cool the extracts to room temperature and subsequently add 150 µl 2.5 M HCl and mix

☐ ☐ Transfer extracts to vials and cap

Table 9: Preparation MMS

×PL	MMS (µg/kg)		Blank extract (µl)	Mixed standard solution (4.4.2) (µl)	Mixed standard solution (4.4.2) (µl)	methanol (4.2.2) (µl)
	OA, DTX, AZA and PTX	YTX		OA, DTX,PTX, and YTX mix	AZA mix	
0	0	0	865	0	0	135
0.125	20	125	865	5	6.3	123.7
0.25	40	250	865	10	12.5	112.5
0.5	80	500	865	20	25.0	90
1	160	1000	865	40	50.0	45
1.5	240	1500	865	60	75.0	0

Table 10: Preparation MMS for hydrolysis

×PL	MMS (µg/kg)	blank extract (µl)	Mixed standard solution OA / DTX1 and DTX2 for hydrolysis (4.4.3) (µl)	methanol (4.2.2) (µl)
	OA / DTX1 / DTX2			
0	0	1850	-	150
0.125	20	1850	12.5	137.5
0.25	40	1850	25	125
0.5	80	1850	50	100
1	160	1850	100	50
1.5	240	1850	150	-

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RIKILT is a member of various national and international expertise centres and networks. Most of our work is commissioned by the Dutch Ministry of Economic Affairs, Agriculture and Innovation and the new Dutch Food and Consumer Product Safety Authority. Other parties commissioning our work include the European Union, the European Food Safety Authority (EFSA), foreign governments, social organisations, and businesses.

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