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## Quantitative determination of marine lipophilic toxins in mussels, oysters and cockles using liquid chromatography - mass spectrometry. Interlaboratory validation study

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Hester J. van den Top, Arjen Gerssen, Pearse Mccarron, Hans van Egmond. Quantitative determination of marine lipophilic toxins in mussels, oysters and cockles using liquid chromatography - mass spectrometry. Interlaboratory validation study. Food Additives and Contaminants, 2011, pp.1. 10.1080/19440049.2011.608382 . hal-00741567

**HAL Id: hal-00741567**

**<https://hal.science/hal-00741567>**

Submitted on 14 Oct 2012

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**Quantitative determination of marine lipophilic toxins in mussels, oysters and cockles using liquid chromatography – mass spectrometry. Interlaboratory validation study**

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2011-238.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	22-Jul-2011
Complete List of Authors:	van den Top, Hester; RIKILT Institute of Food Safety, Wageningen UR Gerssen, Arjen; RIKILT Institute of Food Safety, Wageningen UR McCarron, Pearse; National Research Council, Institute for Marine Biosciences van Egmond, Hans; RIKILT Institute of Food Safety, Wageningen UR,
Methods/Techniques:	LC/MS, Inter-laboratory validation
Additives/Contaminants:	Phycotoxins, DSP, Natural toxicants
Food Types:	Seafood
Abstract:	<p>Thirteen laboratories participated in an interlaboratory study to evaluate the 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 mussel (<i>Mytilus edulis</i>), oyster (<i>Crassostrea gigas</i>) and cockle (<i>Cerastoderma edule</i>) matrices. 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 to the still widely applied biological methods (mouse or rat bioassay).</p> <p>Validation was done according to the AOAC 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 <math>\leq 1.8</math> (median HORRAT: 0.8) in all tested</p>

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	<p>materials. The recoveries of OA-, AZA- and YTX- group toxins were within the range of 80 – 108% and for PTX2 was 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/DTXs, AZAs, YTXs and PTX2 in shellfish.</p>

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3 **Quantitative determination of marine lipophilic**  
4 **toxins in mussels, oysters and cockles using liquid**  
5 **chromatography – mass spectrometry :**  
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7 **Inter-laboratory validation study**  
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### Abstract

Thirteen laboratories participated in an inter-laboratory study to evaluate the 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 mussel (*Mytilus edulis*), oyster (*Crassostrea gigas*) and cockle (*Cerastoderma edule*) matrices. 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 to the still widely applied biological methods (mouse or rat bioassay). Validation was conducted according to the AOAC 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  $\leq 1.8$  (median HORRAT: 0.8) in all tested materials. The recoveries of OA-, AZA- and YTX- group toxins were within the range of 80 – 108% and for PTX2 was 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/DTXs, AZAs, YTXs and PTX2 in shellfish.

**Keywords:** LC-MS/MS, mass spectrometric detection, validation study, lipophilic toxins, marine biotoxins, DSP, okadaic acid, azaspiracid, yessotoxin, pectenotoxin, shellfish

## Introduction

Okadaic acid (OA), dinophysistoxins (DTXs), yessotoxins (YTXs), azaspiracids (AZAs) and pectenotoxins (PTXs) are some of the most predominant marine lipophilic shellfish toxins produced by dinoflagellates. Consumption of filter feeding bivalve molluscs such as mussels, oysters and cockles that have fed on toxic dinoflagellates can lead to intoxications. Diarrhoeic shellfish poisoning (DSP), the human intoxication caused by some of these toxins, is a worldwide problem (Food and Agriculture Organization (FAO) 2004) with adverse effects such as nausea, vomiting, abdominal cramps and diarrhoea. In European Union (EU) legislation, maximum levels (ML) have been established for various marine biotoxins in bivalve molluscs. Bivalve molluscs can be placed on the market for human consumption, if they meet the standards laid down in the regulation (European Parliament 2004). For OA, DTXs (including their esters) and PTXs together the total ML is set at 160  $\mu\text{g}$  of OA equivalents per kg of edible shellfish. The MLs for YTXs and AZAs are set at 1 mg of yessotoxin (YTX) equivalents per kg and 160  $\mu\text{g}$  of azaspiracid-1 (AZA1) equivalents per kg, respectively. Until recently the universally accepted method and reference method in the EU for protecting humans from exposure to these lipophilic toxins through shellfish consumption were the mouse and rat bioassays (European Parliament 2005). Whilst these rodent assays have protected consumers for many decades, there are a number of shortcomings such as a high limit of detection, poor reproducibility and, most importantly, ethical issues associated with the use of live animals for analysis. In recent years the European Food Safety Authority (EFSA) has assessed the current EU limits with regard to human health and the methods of analysis for marine biotoxins. This has resulted in several published opinions (EFSA 2009). EFSA established acute reference doses (ARfD) for several (groups of) marine biotoxins, including the lipophilic toxins. Based on the assessments, EFSA calculated the maximum concentrations of marine biotoxins in shellfish which would ensure that the ARfD would not be exceeded when consuming a single 400 g portion of shellfish meat. These maximum concentrations are 45  $\mu\text{g}$  of OA equivalents per kg and 30  $\mu\text{g}$  of AZA1 equivalents per kg. If the EU decides to lower the ML taking the EFSA opinions into account, this will

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3 have an impact on the methods that can be applied for the determination  
4 of OA-group toxins and AZA-group toxins. For YTXs and PTXs EFSA  
5 calculated that the maximum concentrations in shellfish meat are 3.75 mg  
6 YTX equivalents per kg and 120 µg PTX2 equivalents, to ensure that the  
7 ARfD for these toxins would not be exceeded.  
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12 Furthermore, regulatory developments in the EU are directed towards  
13 replacement of rodent assays for marine lipophilic toxins by LC-MS/MS  
14 methodology in the coming years, at the latest by the end of 2014  
15 (European Parliament 2011).  
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20 Within the EU project 'BIOTOX' (EU's Sixth Framework Programme for  
21 Research and Technological Development) an LC-MS/MS method was  
22 developed and in-house validated at RIKILT Institute of Food Safety, the  
23 Netherlands (Gerssen *et al.* 2009; Gerssen *et al.* 2010). The method  
24 includes a sample preparation using 100% methanol as the extraction  
25 solvent and a hydrolysis procedure (Mountfort *et al.* 2001) to convert the  
26 OA/DTX-esters to the free OA/DTX toxins. The method prescribes the use  
27 of matrix matched standards, both not-hydrolysed and hydrolysed to  
28 correct for matrix effects. The method specifies the use of a analytical LC  
29 column capable of handling pH 11, for example a Waters X-Bridge C18  
30 (150 mm x 3 mm, 5 µm), for the separation of toxins. For elution,  
31 alkaline conditions are used where mobile phase A is water and B is  
32 acetonitrile/water (90:10,v/v) and both contain 6.7 mM ammonium  
33 hydroxide (pH11). In 2009 a limited pre-validation study was organized  
34 involving 4 laboratories from 3 countries. This pre-validation study was  
35 conducted to test the method in an inter-laboratory environment. The  
36 analysis procedure included a sample preparation with and without a solid  
37 phase extraction (SPE) clean-up for optimal reduction of matrix effects.  
38 The pre-validation study yielded satisfactory results (Gerssen 2010) and  
39 gave confidence to undertake a full collaborative exercise in 2010. The  
40 concentrations found with and without the SPE procedure were not  
41 significantly different from each other in this pre-validation study and  
42 therefore it was decided that the SPE procedure would not be included in  
43 the inter-laboratory validation study.  
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5 The primary aim of this inter-laboratory validation study was to assess  
6 the performance characteristics of the method via a collaborative trial  
7 designed according to internationally accepted guidelines for method  
8 validation (Horwitz 1995). The study involved the quantitative  
9 determination of free (before hydrolysis) and total (after hydrolysis) OA-  
10 group toxins [OA, DTX1 and DTX2], AZA-group toxins [AZA1, AZA2 and  
11 AZA3], PTX2 and YTX-group toxins [YTX and 45-hydroxy-yessotoxin (45-  
12 OH-YTX)]. The method performance characteristics for total toxicity for  
13 each toxin group expressed as equivalents was also assessed, because in  
14 the EU legislation the MLs are expressed as OA-, AZA- and YTX-  
15 equivalents (European Parliament 2004).  
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#### 24 *Study design*

25 The study was designed in accordance with the Collaborative Study  
26 Guidelines of AOAC INTERNATIONAL (AOAC 2002). Twenty laboratories  
27 were invited to take part in this study, of which 14 laboratories confirmed  
28 interest in participation. The participants represented countries covering a  
29 large part of the European coastline where lipophilic toxins producing  
30 algae occur. One organisation from the United States of America (USA)  
31 requested to take part in the study and was included as a participant. All  
32 participants had experience in analysing lipophilic toxins, some had  
33 already implemented the alkaline chromatographic conditions, while other  
34 participants were not familiar with these conditions. All laboratories  
35 participated in a training phase where they were supplied with sample  
36 extracts, a detailed standard operating procedure (SOP) and reporting  
37 sheets. The purpose of the training phase was to get experienced with the  
38 alkaline conditions as well as to evaluate if the desired limit of  
39 quantitation [(LOQ) (<40 µg/kg for OA)] could be met. The materials for  
40 the training phase consisted of a matrix matched standard (MMS) series  
41 and a set of spiked and naturally contaminated mussel extracts. Feedback  
42 from the training phase was used to modify the SOP and reporting sheets.  
43 All laboratories met the LOQ criteria, but one laboratory indicated that it  
44 could not participate due to a lack of time. Therefore, 13 of the 14  
45 laboratories were invited to participate in the actual validation study.  
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3 The final SOP was sent to the participants who had 5 weeks to analyse a  
4 set of 25 samples composed of eight blind duplicate naturally  
5 contaminated shellfish homogenates, blank shellfish homogenates, spiked  
6 shellfish homogenates and a pre-release freeze-dried certified reference  
7 material (CRM-FDMT1) provided by the National Research Council (NRC,  
8 Halifax, Canada). Criteria for the acceptance of results were that all  
9 calibration curves should have a correlation (R) above 0.980 and that the  
10 slope difference of calibration curves injected before and after a sample  
11 series should be below 25.0%. All sample extracts were analysed by  
12 single injection on the LC-MS/MS.  
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## 20 **Material and methods**

### 21 *Statistics*

22 The statistical approach to evaluate the results of the study was selected  
23 according to internationally accepted guidelines for method evaluation  
24 (Horwitz 1995). The study was based on the principle that participants  
25 analysed a set of samples which consists of blind duplicates. The samples  
26 were shellfish homogenates containing the marine lipophilic toxins under  
27 investigation in this study, at different concentration levels. Quantitative  
28 results submitted by participants were used to estimate the average  
29 concentrations and standard deviations under repeatability and  
30 reproducibility conditions.  
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40 When participants did not fulfil the criteria ( $R > 0.980$  and slope difference  
41  $< 25.0\%$  for the calibration curves) the reported data were considered to  
42 be "not valid" and were excluded from statistical evaluation. Following the  
43 AOAC guidelines (AOAC 2002), all the outliers have to be flagged and  
44 excluded by the harmonised outlier removal procedure. This procedure  
45 consists of sequential application of the Cochran and Grubbs tests (at  
46 2.5% probability level, one-tail for Cochran and two-tailed for Grubbs)  
47 until no further outliers are flagged. Outlier detection stops before a  
48 maximum drop of 22.2% in the original number of laboratories providing  
49 valid data occurs (AOAC 2002).  
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The precision data obtained from the inter-laboratory study were compared with the predicted acceptable errors for precision. These predicted errors were calculated from the Horwitz equation (Horwitz *et al.* 1980), which represents an empirical relation between the acceptable precision and the corresponding toxin concentration in the sample analysed. The precision calculated with this equation provides an indication whether the method is sufficiently precise for the concentration level of the toxin being measured. This indication is expressed by the HORRAT (Horwitz and Albert 2006). For the between-laboratory reproducibility, the HORRAT is a ratio between the measured relative standard deviation for reproducibility ( $RSD_R$ ) and the predicted relative standard deviation for reproducibility ( $PRSD_R$  calculated by Horwitz equation).

$$HORRAT = \frac{RSD_R}{PRSD_R} \text{ [equation 1]}$$

where

$$PRSD_R = 2^{(1-0.5\text{Log}C)} (\approx 2C^{-0.1505}) \text{ [equation 2]}$$

C in [equation 2] is the estimated mean concentration expressed as a decimal fraction. In this study, the decimal fraction for each material was calculated as:  $C = (\text{mean } \mu\text{g toxin/kg}) \text{ multiplied by } 10^{-9}$ . The target value for the HORRAT is set at 1; HORRAT values  $<1.5$  are considered acceptable, between 1.5 and 2.0 is considered as rather acceptable and a HORRAT  $>2.0$  is considered to be not acceptable.

#### Standards

CRMs for OA (CRM-OA-c –  $17.7 \pm 1.8 \mu\text{mol/L}$ ), AZA1 (CRM-AZA1 –  $1.47 \pm 0.08 \mu\text{mol/L}$ ), PTX2 (CRM-PTX2 –  $10.0 \pm 0.4 \mu\text{mol/L}$ ) and YTX (CRM-YTX + isomer –  $4.6 \pm 0.2 \mu\text{mol/L}$ ) were obtained from the NRC. Dilutions of these CRMs were used for the preparation of spiked shellfish homogenates and for the preparation of spiking solutions.

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3 For this study, the NRC made pre-release CRMs available for the  
4 individual toxins AZA2 (1.5  $\mu\text{mol/L}$ , containing trace amounts of AZA1 and  
5 AZA3, <0.2% relative to AZA2), AZA3 (1.4  $\mu\text{mol/L}$ , containing some  
6 isomeric impurities of AZA3 and AZA4, <5.9% relative to AZA3), DTX1  
7 (18.7  $\mu\text{mol/L}$ , containing approx 0.5% in total of DTX1 isomer impurities)  
8 and DTX2 (9.7  $\mu\text{mol/L}$ , containing a trace amount of OA <0.5 %).

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14 The coordinating laboratory purchased ampoules of these pre-release  
15 CRMs for the inter-laboratory study and provided them to the participants.  
16 Incorporating these CRMs made it possible to quantify the toxins in  
17 samples against a full calibration curve of the corresponding toxin. At the  
18 time of this study these pre-release CRMs were not yet commercially  
19 available. A common practice which is applied when no CRM is available  
20 for an individual toxin is to quantify the toxin against an available CRM  
21 from that specific toxin group (e.g. DTX1 against OA). In order to  
22 evaluate the effects of quantifying against the different calibrants the  
23 reporting sheets were designed such that quantification was done both  
24 against the corresponding individual toxin as well as against the  
25 corresponding toxin group representative.  
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### 35 *Test materials*

36 For the inter-laboratory validation study eight toxin-containing shellfish  
37 materials were provided to the participants. Beside these toxin-containing  
38 samples participants were also provided with two blank materials and two  
39 spiked materials making a total of 23 shellfish homogenate samples to be  
40 tested in this validation study. Additionally two portions of a pre-release  
41 CRM-FDMT1 were included in the sample set sent to participants  
42 (McCarron 2011a). Five mussel (*Mytilus edulis*) materials, two oyster  
43 (*Crassostrea gigas*) materials and one cockle (*Cerastoderma edule*)  
44 material were prepared by mixing blank tissue with naturally  
45 contaminated shellfish homogenates of the same matrix to achieve a  
46 toxin level around the current ML. The blank shellfish materials were  
47 obtained within the Dutch monitoring program for marine biotoxins.  
48 Homogenates of blank mussels, oysters and cockles were prepared by  
49 homogenizing whole flesh tissue with a T25 Ultra Turrax mixer at 24,000  
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rpm for 10 – 15 min. Mussel materials containing OA-group toxins (free and ester forms) and AZA-group toxins were kindly donated by C. Duffy and S. Burrell, Marine Institute, Oranmore, Ireland. Mussel materials containing YTX and 45-OH-YTX were kindly donated by Dr. J.A.B. Aasen, Norwegian School of Veterinary Science, Oslo, Norway. Mussels containing OA group toxins were kindly provided by Dr. K. Jørgensen, Technical University of Denmark, National Food Institute, Division of Food Chemistry, Copenhagen, Denmark and Dr. A. These, BfR Federal Institute for Risk Assessment, Berlin, Germany. Mussel materials with OA-group toxins, YTX-group toxins and PTX2 were kindly donated by Dr. S. Morris, CEFAS, Weymouth, United Kingdom. Unfortunately, for oyster and cockle materials it was not possible to obtain naturally contaminated materials. 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% for the two oyster materials and the cockle material, respectively. All materials were included in the study as blind duplicates.

To assess the trueness of the method (expressed as recovery), two blank mussel homogenates were spiked. One mussel homogenate was spiked to a level of 100 µg/kg OA, 48 µg/kg AZA1 and 300 µg/kg YTX and a second mussel homogenate to a level of 100 µg/kg OA and 48 µg/kg AZA1. Participants were provided with a freeze-dried pre-release reference material (FDMT1, NRC) and specific instructions how to reconstitute prior to extraction. FDMT1 contained a broad spectrum of toxins at the following (preliminary certified) levels expressed as reconstituted wet tissue concentrations: 275 µg/kg OA, 133 µg/kg DTX1, 595 µg/kg DTX2, 712 µg/kg AZA1, 198 µg/kg AZA2, 186 µg/kg AZA3, 119 µg/kg PTX2 and 450 µg/kg YTX. All participants were accurate and precise (within stated limits) with the quantities of dry material (powder) and water used in the reconstitution process. The homogeneity and stability of the FDMT1 material has been well established (McCarron 2011b). Furthermore, participants were requested to purchase and analyse the CRM-Mus-B material (NRC), containing 10.1 µg/g OA and 1.3 µg/g DTX1. Because of potential stability issues for PTX2 in shellfish homogenates (Miles *et al.*

2004), laboratories were provided with 5 vials each containing  $100 \pm 0.3$   $\mu$ l of PTX2 solutions at two different concentrations which they had to add quantitatively to 1.0 gram of specifically numbered samples to obtain levels of 100 and 200  $\mu$ g/kg, respectively.

The blank, contaminated and spiked materials were mixed using an Ultra Turrax mixer for 10-15 min at 24,000 rpm. At least 150 g of each material was prepared, small test portions of 1.5 g from each material were dispensed in plastic test tubes. From each material 10 samples were taken for homogeneity testing. At the start and during the study subsets were set aside at -80 °C for stability testing. The remaining test portions of each material were randomly numbered with individual codes for each participant.

Homogeneity tests were performed on the prepared materials at RIKILT according to the International Organization for Standardization (ISO) guide 13528:2005 (ISO 2005) and the materials were shown to be sufficiently homogenous [ $RSD_R(\%)$  for 10 subsamples  $<0.3 \times PRSD_R(\%)$ ].

A blank mussel and a blank oyster material were also included in the test sample set. One of the blank oyster material duplicate samples was marked as "blank oyster" and participants were requested to prepare from this matrix a methanolic extract and spike it at  $0.5 \times ML$  (maximum level, as established by EU legislation). This test was intended as a check for possible differences in matrix effects for oyster and mussel extracts that participants used for preparation of the MMS.

#### *Calibration curves*

MMS series were used to construct calibration curves. Each participant prepared an extract from a blank mussel homogenate. This blank mussel homogenate was not provided for the study and therefore participants had to obtain their own blank material. The blank methanolic extracts were used to prepare MMS. The MMS series represented 0, 0.125, 0.25, 0.5, 1 and 1.5 times the current ML (table 1). For the measurement of

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3 hydrolysed extracts an additional hydrolysed MMS series was prepared  
4 which contained only OA group toxins.  
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#### 7 8 *LC-MS/MS analysis*

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10 The LC-MS/MS method applied was previously described and in-house  
11 validated (Gerssen *et al.* 2009; Gerssen *et al.* 2010). The method includes  
12 a sample preparation using 100% methanol as the extraction solvent and  
13 a hydrolysis procedure (Mountfort *et al.* 2001) to convert the OA/DTX-  
14 esters to the free OA/DTX toxins. MMS, both non-hydrolysed and  
15 hydrolysed, are included in the method to correct for matrix effects. The  
16 method specifies the use of a analytical LC column capable of handling pH  
17 11, for example a Waters X-Bridge C18 (150 mm x 3 mm, 5 µm), for the  
18 separation of toxins. For elution, alkaline conditions are used where  
19 mobile phase A is water and B is acetonitrile/water (90:10,v/v) and both  
20 contain 6.7 mM ammonium hydroxide (pH11). If an alternative analytical  
21 column to the one specified had to be used it was required that this  
22 alternative column showed a comparable elution order of the toxins as  
23 described by Gerssen *et al.* (2009). The mass spectrometric settings for  
24 ionization as well as fragmentation were optimized at each participant's  
25 laboratory. The ionization polarity was prescribed; for OA-group toxins as  
26 well as YTX-group toxins negative electrospray ionization (ESI<sup>-</sup>) should be  
27 applied and for AZA-group toxins and PTX2 positive ESI (ESI<sup>+</sup>). The  
28 injection order of the samples and calibration curves was prescribed as:  
29 MMS, then the sample extracts and then a second injection of the MMS.  
30 The SOP prescribed that sample extracts were injected only once.  
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## 45 **Results and discussion**

### 46 *Training phase*

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48 In the training phase, the participants were asked to determine the  
49 concentrations of the marine lipophilic toxins OA, AZA1, PTX2 and YTX in  
50 the provided mussel extracts. Participants were also provided with a  
51 ready-prepared MMS series in blank mussel extracts. Thirteen out of the  
52 14 laboratories reported results. Participants had to provide precise  
53 information about the LC-MS/MS conditions applied as well as  
54 representative chromatograms. Some participants used alternative HPLC  
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3 columns to the XBridgeC<sub>18</sub> (e.g. Gemini NX; Gemini C<sub>18</sub>; AQCUIITY UPLC  
4 BEH C<sub>18</sub>) but no particular problems were observed in the elution order.  
5 One laboratory obtained questionable results because calibration curves  
6 did not fulfil the required criteria and substantial divergent results for OA  
7 were reported. The laboratory had deviated from the SOP by adapting the  
8 HPLC conditions to some extent. The laboratory reanalysed the extracts  
9 three times and ultimately obtained calibration curves that fulfilled the  
10 criteria. Despite the questionable initial performance of this laboratory, it  
11 was accepted for participation in the main study.  
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19 For the extract of a naturally contaminated material that contained a wide  
20 variety of toxins, indicative calculated HORRATs were all below 1.7.  
21 Therefore, all laboratories that delivered results in the training phase were  
22 accepted to participate in the inter-laboratory study.  
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#### 27 *Inter-laboratory study.*

28 In total, 13 laboratories provided results for the full inter-laboratory  
29 study that involved the quantitative determination of OA, DTX1, DTX2,  
30 AZA1, AZA2, AZA3, PTX2, YTX and 45-OH-YTX in 23 shellfish  
31 homogenates and two portions of FDMT1. After initial inspection of all  
32 results and verifying whether the participants applied the SOP correctly,  
33 one laboratory was excluded owing to misidentified toxins. These  
34 misidentifications might be due to changes in the gradient applied (3 x  
35 steeper than described in the SOP). It was also noticed that data were  
36 missing in the reporting sheets. Because of these observations, all results  
37 of this participant were considered to be "not-valid" and were excluded for  
38 statistical analysis. Mass spectrometer instruments used by participants  
39 included the Applied Biosystems QTrap 3200, 4000, 5500 and the  
40 API4000, Waters Quattro Ultima, Quattro Micro and Quattro Premier,  
41 Agilent 6460 triple quad and Varian 1200L triple quad. When participants  
42 did not fulfil the criteria for the calibration curve to quantify the samples,  
43 i.e. a calibration curve slope difference above 25.0% and/or a correlation  
44 below 0.980, the results were considered "not-valid" and were not  
45 included in the statistical analysis. Participants had the opportunity to split  
46 the samples over two separate series (days), therefore it was possible  
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3 that for one day the criteria were met, and for the other day the criteria  
4 were not met. This was the case with one participant who experienced  
5 problems with the LC-MS/MS system. Only results from days on which the  
6 criteria were met were accepted as valid results and included in the  
7 statistical evaluation. All participants reported the limit of detection (LOD)  
8 and limit of quantification (LOQ) for all individual toxins in the reporting  
9 sheets. From the reported values the averages were calculated and  
10 presented in table 2. The LOQ for all toxins is well below the current MLs  
11 and will suffice in the event of MLs being lowered in the future. Statistical  
12 analysis was not performed on the materials which had toxin levels below  
13 the LOQ. The spiked oyster extract at a level of  $0.5 \times \text{ML}$  revealed that  
14 there were no differences in matrix effects for the oyster and mussel  
15 extracts that participants used for preparation of the MMS. Results  
16 obtained with the  $0.5 \times \text{ML}$  level in blank oyster extract showed that for all  
17 toxins used in the MMS the results in oyster extract were  $96 \pm 4.6 \%$  of  
18 the expected concentration (table 3).  
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30 After the exclusion of the "not-valid" data, valid data were tested for  
31 outliers by applying the Cochran and Grubbs tests. Details about which  
32 laboratories were excluded can be found in tables 4,5,6 and 7. For all  
33 toxins, less than 22.2% of the laboratories were identified as outliers. In  
34 tables 4 and 5, details are given about the validation results obtained for  
35 the OA-group toxins (including their esters) and AZA-group toxins  
36 respectively, quantified against the corresponding toxins. In table 6  
37 results are presented for PTX2 and the individual YTX-group toxins. In the  
38 range from 0.125 to 2 times the current MLs, HORRATs in all tested  
39 materials for the individual toxins (except for 45-OH-YTX) were found to  
40 be below or equal to 1.8 (median HORRAT: 0.8). For 45-OH-YTX toxins  
41 (the only toxin for which no individual calibrant was available) HORRATs  
42 for two materials are higher, 2.35 for material 2 (mussel) and 1.81 for  
43 material 7 (cockle). Tables 4, 5 and 6 list the 8 naturally contaminated  
44 materials used in this study including the toxins, grand mean  
45 concentrations and performance characteristics found.  
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56 Additionally, the method performance characteristics were established for  
57 the total toxicity of the OA-group toxins and the AZA-group toxins  
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3 quantified against the individual calibrants available and using the toxicity  
4 equivalency factors (TEFs) as published by EFSA (EFSA 2009). For YTX-  
5 group toxins the method performance characteristics were established for  
6 the total toxicity against YTX. For calculations of the total toxicity method  
7 performance characteristics, only valid results from the individual toxins  
8 were taken into account for the total toxicity calculations. In instances  
9 where (one of) the individual toxin result was considered as "not-valid",  
10 the laboratory result for total toxicity was considered to be "not-valid" and  
11 not included in the statistical evaluation. When a laboratory had a valid  
12 result but was an "outlier" for one of the individual toxins (Cochran or  
13 Grubbs test) the result was taken into account for the statistical  
14 evaluation of the total toxicity. Results for the total toxicity method  
15 performance for the OA-, AZA- and YTX-group are presented in tables 4 –  
16 6 respectively.

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19 The HORRATs for the total toxicity (including YTX-group) are all below 1.8.  
20 Based on these findings it was concluded that the method is fit for the  
21 intended purpose.

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24 In addition, the results of the study were also utilised to assess the  
25 trueness of the method. This was possible since test materials provided to  
26 the participants included spiked homogenates and (pre-release) certified  
27 reference materials. As an indicator of the trueness of the method, the  
28 percentage ratios of the grand mean values obtained in the validation  
29 study to the target concentrations of the individual toxins are presented in  
30 table 7. Recovery values are within the expected range of 80 – 110%,  
31 with the exception of the PTX2 result in the pre-release reference material  
32 where the recovery was relatively low (62%).

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35 To indicate a decision limit at which level a sample can be considered to  
36 contain a toxin level above the ML, based on the average HORRAT values  
37 obtained for each individual toxin, the average method  $RSD_R$  was  
38 calculated.

$$Avg_{HORRAT} = \frac{RSD_{R\_obtained}}{PRSD_R} \text{ [equation 3]}$$

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5 The  $PRSD_R$  at the PL was obtained from Horwitz equation [equation 2].  
6 The average within lab reproducibility ( $RSD_r$ ) was derived from the "RSD<sub>R</sub>  
7 Obtained" by dividing the value by  $2\sqrt{2}$ . The calculation of the decision  
8 limit  $CC\alpha$  (95% confidence interval) at ML was calculated by  
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$$11 \quad CC\alpha = ML + (1.64 \times (ML \times RSD_r)) \text{ [equation 4]} \\ 12$$

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16 In which ML is the permitted level for the toxins in  $\mu\text{g}/\text{kg}$ , The value 1.64  
17 is from a one-tailed t distribution with  $P=0.05$  (infinite number degrees of  
18 freedom). The decision limits as obtained from these calculations are  
19 presented in table 2. In this study this approach showed that in the event  
20 a mass fraction of a toxin in a sample is found at or above the  $CC\alpha$  it  
21 indicates with a probability of  $1-\alpha$ , or 95% ( $\alpha=5\%$ ), that the sample is  
22 above the ML.  
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#### 29 *Further observations*

30 Because the study involved quantification of the lipophilic toxins both  
31 against the individual calibrants and against analogues, a comparison was  
32 made to see if there was a difference in the quantitative results both for  
33 the reported concentrations and the method precision. Results of this  
34 comparison for the toxins DTX1, DTX2, AZA2 and AZA3 are presented in  
35 table 8. When comparing the results of DTX1 quantified against OA with  
36 DTX1 quantified against DTX1 (table 8) it can be seen that for all  
37 materials the DTX1 content is estimated to be 1.2 x higher when  
38 quantified against DTX1 compared to quantification against OA ( $n=6$ , c.v.  
39 7.5 %). A similar effect is observed for the results of DTX1 after  
40 hydrolysis where the DTX1 content is estimated to be 1.4 x higher when  
41 quantified against DTX1 than when quantified against OA ( $n=6$ , c.v.  
42 5.1%). The same is the case for AZA3. It can be seen that the AZA3  
43 content quantified against AZA3 is about 1.5 x higher ( $n=7$ , c.v. 4.7%),  
44 when compared with AZA3 quantified against AZA1. For DTX2 and AZA2  
45 the effect is less noticeable. The practical approach to quantify a toxin  
46 against the only commercially available analogue may cause a systematic  
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3 error in the trueness. The method precision (HORRATs) is in most cases  
4 equal or better when the quantification is done against the individual  
5 corresponding calibrant. The assumption that the response in the mass  
6 spectrometer of e.g. OA is equal to the response of DTX1 and thus DTX1  
7 can be quantified against either OA or DTX1 with the same precision and  
8 trueness, may not be true. This is not a shortcoming of the method but is  
9 a consequence of the approach when the individual corresponding  
10 calibrant is not available and the closest comparable analogue is used for  
11 quantification.  
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### 21 **Conclusion**

22 The LC-MS/MS method for the simultaneous determination of the  
23 lipophilic toxins OA, DTX1, DTX2, AZA1, AZA2, AZA3, YTX, 45-OH-YTX  
24 and PTX2 has been successfully validated in an inter-laboratory study.  
25 Results of the validation process confirmed its fitness for purpose as a  
26 quantitative method, since all HORRATs in the relevant range were found  
27 to be  $\leq 1.8$  (median HORRAT: 0.8). Trueness (expressed as recovery) for  
28 OA, AZA and YTX group toxins was found to be within the range of 80 –  
29 108% and for PTX2 within the range of 62 – 93%. Based on these values  
30 for precision and recovery, it was concluded that the method is suitable  
31 for official control purposes to quantitatively determine OA/DTXs, AZAs,  
32 YTXs and PTX2 in shellfish.  
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### 42 **Acknowledgements**

43 We would like to thank the participation institutes: CER - Centre  
44 d'Economie Rurale (Marloie, Belgium), IPH - Scientific Institute of Public  
45 Health (Brussels, Belgium), IFREMER - Institut français de recherche pour  
46 l'exploitation de la mer (Nantes, France), BfR - Bundesinstitut für  
47 Risikobewertung (Berlin, Germany), Chemical and Veterinary Analytical  
48 Institute (Muenster, Germany), Marine Institute (Oranmore, Ireland),  
49 Centro Ricerche Marine (Cesenatico, Italy), Norwegian School of  
50 Veterinary Science (Oslo, Norway), IRTA - Agencia Española de Seguridad  
51 Alimentaria y Nutrición (Tarragona, Spain), CEFAS - Centre for  
52 Environment, Fisheries & Aquaculture Science (Weymouth, United  
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3 Kingdom), AFBI – Agri-Food and Biosciences Institute (Belfast, United  
4 Kingdom), NOAA – National Oceanic and Atmospheric Administration  
5 (Charleston, USA). We would like to thank the EU-RL on Marine Biotoxins  
6 (Vigo, ES), because of their continuous support in the preparation and  
7 organisation of this study.  
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### 11 12 13 **References**

14 AOAC International. 2002. AOAC Guidelines for collaborative study  
15 procedures to validate characteristics of a method of analysis Appendix D.  
16  
17

18  
19 European Food Safety Authority (EFSA). 2009. Marine biotoxins in  
20 shellfish – Summary on regulated marine biotoxins, Scientific Opinion of  
21 the Panel on Contaminants in the Food Chain. The EFSA Journal. 1306:1-  
22 23.  
23  
24  
25

26  
27 European Parliament. 2004. Regulation (EC) No 853/2004 of the  
28 European Parliament and of the Council of 29 April 2004 laying down  
29 specific hygiene rules for food of animal origin. Official Journal of the  
30 European Union. L139:55 - 205.  
31  
32  
33

34  
35 European Parliament. 2005. Commission Regulation (EC) No 2074/2005  
36 of 5 December 2005 laying down implementing measures for certain  
37 products under Regulation (EC) No 853/2004 of the European Parliament  
38 and of the Council and for the organisation of official controls under  
39 Regulation (EC) No 854/2004 of the European Parliament and of the  
40 Council and Regulation (EC) No 882/2004 of the European Parliament and  
41 of the Council, derogating from Regulation (EC) No 852/2004 of the  
42 European Parliament and of the Council and amending Regulations (EC)  
43 No 853/2004 and (EC) No 854/2004 (Text with EEA relevance). Official  
44 Journal of the European Union. L388:27 - 44.  
45  
46  
47  
48  
49  
50  
51

52  
53 European Parliament. 2011. Regulation (EC) no. 15/2011 of 10 January  
54 2011 amending Regulation (EC) No 2074/2005 as regards recognised  
55 testing methods for detecting marine biotoxins in live bivalve molluscs.  
56 Official Journal of the European Union. L6:3.  
57  
58  
59  
60

1  
2  
3  
4 Food and Agriculture Organization (FAO). 2004. Marine biotoxins. FAO  
5 Food and Nutrition Paper 80. Food and Agricultural Organization of the  
6 United Nations. Rome. Italy.  
7  
8

9  
10  
11 Gerssen A. 2010. The analysis of lipophilic marine toxins [dissertation].  
12 [Wageningen, The Netherlands]: Wageningen University  
13

14  
15  
16 Gerssen A, Mulder PPJ, McElhinney MA, De Boer J. 2009. Liquid  
17 chromatography - tandem mass spectrometry method for the detection of  
18 marine lipophilic toxins under alkaline conditions. Journal of  
19 Chromatography A. 1216:1421-1430.  
20  
21

22  
23  
24 Gerssen A, van Olst EH, Mulder PP, de Boer J. 2010. In-house validation  
25 of a liquid chromatography tandem mass spectrometry method for the  
26 analysis of lipophilic marine toxins in shellfish using matrix-matched  
27 calibration. Analytical and Bioanalytical Chemistry. 397:3079-88.  
28  
29

30  
31  
32 Horwitz W. 1995. Protocol for the design, conduct and interpretation of  
33 method-performance studies: Revised 1994 (Technical Report). Pure Appl.  
34 Chem. 67:331-343.  
35  
36

37  
38  
39 Horwitz W, Albert R. 2006. The Horwitz ratio (HORRAT): a useful index of  
40 method performance with respect to precision. J AOAC Int. 89:1095-1109.  
41  
42

43  
44  
45 Horwitz W, Albert R, Kamps L, Boyer K. 1980. Quality assurance in the  
46 analysis of foods and trace constituents. J Assoc Off Anal Chem. 67:1344-  
47 1354.  
48

49  
50 International Organization for Standardization (ISO). 2005. ISO-Guide.  
51 13528:2005. Statistical methods for use in proficiency testing by  
52 interlaboratory comparisons. International Organization for  
53 Standardization. Geneva. Switzerland.  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 McCarron P, Emteborg H, Nulty C, Rundberget T, Loader JI, Teipel, K,  
4 Miles CO, Quilliam, MA, Hess P. 2011a. A mussel tissue certified reference  
5 material for multiple phycotoxins. Part 1: design and preparation.  
6 Analytical and Bioanalytical Chemistry. 400:821-833.  
7  
8

9  
10  
11 McCarron P, Emteborg H, Nulty C, Rundberget T, Loader JI, Teipel, K,  
12 Miles CO, Quilliam, MA, Hess P. 2011b. A mussel tissue certified reference  
13 material for multiple phycotoxins. Part 3: homogeneity and stability.  
14 Analytical and Bioanalytical Chemistry. 400:847-858.  
15  
16

17  
18  
19 Miles CO, Wilkins AL, Munday R, Dines MH, Hawkes AD, Briggs LR,  
20 Sandvik M, Jensen DJ, Cooney JM, Holland PT, Quilliam MA, MacKenzie AL,  
21 Beuzenberg V, Towers NR. 2004. Isolation of pectenotoxin-2 from  
22 Dinophysis acuta and its conversion to pectenotoxin-2 seco acid, and  
23 preliminary assessment of their acute toxicities. Toxicon. 43:1 - 9.  
24  
25  
26

27  
28  
29 Mountfort DO, Suzuki T, Truman P. 2001. Protein phosphatase inhibition  
30 assay adapted for determination of total DSP in contaminated mussels.  
31 Toxicon. 39:383-390.  
32  
33  
34  
35  
36  
37  
38  
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3 Table 1: MMS series concentration range  
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5 Table 2: Average LOD / LOQ reported by participants for the different  
6 toxins based on the quantification on the individual toxin (n=12) and  
7 calculated decision limit  
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9 Table 3: Oyster extract spiked at 0.5 x ML quantified against MMS series  
10 prepared in blank mussel extract  
11

12 Table 4: Method performance characteristics for OA-group toxins in  
13 mussel, oyster and cockle, quantified against the individual corresponding  
14 toxin calibrants  
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16 Table 5: Method performance for AZA-group toxins in mussel, oyster and  
17 cockle, quantified against the individual corresponding toxin calibrant  
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19 Table 6: Method performance for PTX2 and YTX-group toxins in mussels,  
20 oysters and cockle, quantified against PTX2 and YTX respectively  
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22 Table 7: Results for individual toxins to assess trueness based on  
23 quantification against the individual corresponding toxin calibrants  
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25 Table 8: Comparison of toxin quantification against corresponding  
26 calibrant and close analogue calibrant  
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Table 1: MMS series concentration range

×ML	MMS (µg/kg)	
	OA, DTX, AZA and PTX	YTX
0	0	0
0.125	20	125
0.25	40	250
0.5	80	500
1	160	1000
1.5	240	1500

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Table 2: Average LOD / LOQ reported by participants for the different toxins based on the quantification on the individual toxin (n=12) and calculated decision limit

	Average LOD ( $\mu\text{g}/\text{kg}$ )	Average LOQ ( $\mu\text{g}/\text{kg}$ )	CC $\alpha$ (95% confidence interval) ( $\mu\text{g}/\text{kg}$ )	CC $\alpha$ (95% confidence interval) hydrolysed ( $\mu\text{g}/\text{kg}$ )
<b>OA</b>	3	9	178	184
<b>DTX1</b>	5	12	173	178
<b>DTX2</b>	3	9	176	186
<b>AZA1</b>	2	4	172	
<b>AZA2</b>	2	4	169	
<b>AZA3</b>	3	7	168	
<b>PTX2</b>	2	5	177	
<b>YTX</b>	16	53	1100	

Table 3: Oyster extract spiked at 0.5 x ML quantified against MMS series prepared in blank mussel extract

	Mean value (µg/kg)	# labs with valid data*	Target value (µg/kg)	Recovery (%)	RSDR (%)
<b>OA</b> oyster spike at 0.5 ML	74.9	8	80.0	<b>93.7</b>	20.2
<b>DTX1</b> oyster spike at 0.5 ML	76.5	8	80.0	<b>95.6</b>	14.2
<b>DTX2</b> oyster spike at 0.5 ML	73.1	8	80.0	<b>91.4</b>	15.0
<b>AZA1</b> oyster spike at 0.5 ML	71.1	8	80.0	<b>88.9</b>	28.7
<b>AZA2</b> oyster spike at 0.5 ML	80.8	8	80.0	<b>101.1</b>	12.3
<b>AZA3</b> oyster spike at 0.5 ML	79.0	9	80.0	<b>98.7</b>	14.2
<b>PTX2</b> oyster spike at 0.5 ML	81.7	8	80.0	<b>102.1</b>	21.7
<b>YTX</b> oyster spike at 0.5 ML	486.1	8	500.0	<b>97.2</b>	6.4

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

Table 4: Method performance characteristics for OA-group toxins in mussel, oyster and cockle, quantified against the individual corresponding toxin calibrants

Shellfish matrix		Materials							
		1 Mussel	2 Mussel	3 Mussel	4 Oyster	5 Mussel	6 Mussel	7 Cockle	8 Oyster
OA + esters	No. of labs a(b)	9(0)	9(0)	9(0)	9(0)	9(0)	9(0)	9(0)	9(0)
	Mean concentration (µg/kg)	137.8	66.0	177.6	249.0	618.3 <sup>a</sup>	74.2	976.9 <sup>a</sup>	243.3
	S <sub>r</sub> (µg/kg)	7.81	3.66	22.91	12.60	45.11	7.51	292.05	14.87
	RSD <sub>r</sub> (%)	5.7	5.6	12.9	5.1	7.3	10.1	29.9	6.1
	S <sub>R</sub> (µg/kg)	32.93	16.27	44.25	68.54	192.53	17.40	284.89	55.47
	RSD <sub>R</sub> (%)	23.9	24.7	24.9	27.5	31.1	23.5	29.2	22.8
	<b>HORRAT</b>	<b>1.11</b>	<b>1.02</b>	<b>1.20</b>	<b>1.40</b>	<b>1.81<sup>a</sup></b>	<b>0.99</b>	<b>1.82<sup>a</sup></b>	<b>1.15</b>
DTX1 + esters	No. of labs a(b)	11(0)	11(0)	11	11	10(0)	10	9(1) <sup>b</sup>	10(0)
	Mean concentration (µg/kg)	55.3	98.2	<LOD	<LOD	137.9	<LOD	188.4	33.7
	S <sub>r</sub> (µg/kg)	6.78	9.55	-	-	9.69	-	10.60	3.73
	RSD <sub>r</sub> (%)	12.3	9.7	-	-	7.0	-	5.6	11.1
	S <sub>R</sub> (µg/kg)	13.64	23.25	-	-	26.74	-	33.53	7.20
	RSD <sub>R</sub> (%)	24.6	23.7	-	-	19.4	-	17.8	21.4
	<b>HORRAT</b>	<b>1.00</b>	<b>1.04</b>	-	-	<b>0.90</b>	-	<b>0.87</b>	<b>0.80</b>
DTX2 + esters	No. of labs a(b)	10(0)	10(0)	10(0)	10(0)	11	11(0)	11(0)	11(0)
	Mean concentration (µg/kg)	182.1	9.4 <sup>c</sup>	105.6	251.5	<LOD	13.5	233.3	160.0
	S <sub>r</sub> (µg/kg)	12.84	1.70	16.65	17.83	-	5.56	56.64	19.06
	RSD <sub>r</sub> (%)	7.1	18.1	15.8	7.1	-	41.1	25.1	11.9
	S <sub>R</sub> (µg/kg)	36.93	6.18	28.78	70.17	-	6.68	68.53	43.49
	RSD <sub>R</sub> (%)	20.3	65.9	27.3	27.9	-	49.4	29.4	27.2
	<b>HORRAT</b>	<b>0.98</b>	<b>2.04<sup>c</sup></b>	<b>1.21</b>	<b>1.42</b>	-	<b>1.62</b>	<b>1.47</b>	<b>1.29</b>
OA group total toxicity (µg OA eq./kg)	No. of labs a(b)	9(0)	9(0)	9(0)	9(0)	9(0)	9(0)	9(0)	9(0)
	Mean concentration (µg OA eq./kg)	299.0	168.4	243.4	401.3	757.2	84.9	1293.4	371.4
	S <sub>r</sub> (µg OA eq./kg)	12.00	9.35	33.09	17.80	49.47	7.27	372.44	19.40
	RSD <sub>r</sub> (%)	4.0	5.6	13.6	4.4	6.5	8.6	28.8	5.2
	S <sub>R</sub> (µg OA eq./kg)	65.90	40.42	61.02	110.43	215.37	19.83	355.71	84.60
	RSD <sub>R</sub> (%)	22.0	24.0	25.1	27.5	28.4	23.4	27.5	22.8
	<b>HORRAT</b>	<b>1.15</b>	<b>1.15</b>	<b>1.27</b>	<b>1.50</b>	<b>1.70</b>	<b>1.01</b>	<b>1.79</b>	<b>1.23</b>

a(b) = "a" is number of laboratories remaining after removal of number of outliers indicated by (b); S<sub>r</sub> standard deviation of the repeatability; S<sub>R</sub> standard deviation of the reproducibility; <sup>a</sup>) mass fraction > two times current ML; <sup>b</sup>) lab 21 outlier Cochran's test; <sup>c</sup>) mass fraction is at LOQ (for LOD / LOQ different toxins see table 2)

Table 5: Method performance for AZA-group toxins in mussel, oyster and cockle, quantified against the individual corresponding toxin calibrant

Shellfish matrix		Materials							
		1 Mussel	2 Mussel	3 Mussel	4 Oyster	5 Mussel	6 Mussel	7 Cockle	8 Oyster
AZA1	No. of labs a(b)	11(0)	11	11(0)	11(0)	10	10(0)	10(0)	10
	Mean concentration (µg/kg)	71.0	<LOQ	115.5	194.5	<LOD	252.2	142.4	<LOD
	S <sub>r</sub> (µg/kg)	7.95	-	3.25	5.72	-	8.38	3.85	-
	RSD <sub>r</sub> (%)	11.2	-	2.8	2.9	-	3.3	2.7	-
	S <sub>R</sub> (µg/kg)	12.32	-	10.55	23.70	-	25.60	16.25	-
	RSD <sub>R</sub> (%)	17.3	-	9.1	12.2	-	10.2	11.4	-
	<b>HORRAT</b>	<b>0.73</b>	-	<b>0.41</b>	<b>0.60</b>	-	<b>0.52</b>	<b>0.53</b>	-
AZA2	No. of labs a(b)	12(0)	12	12(0)	12(0)	12	12(0)	11(1) <sup>a</sup>	12
	Mean concentration (µg/kg)	17.2	<LOD	27.5	44.6	<LOD	59.8	35.5	<LOD
	S <sub>r</sub> (µg/kg)	1.82	-	0.81	2.51	-	4.98	2.03	-
	RSD <sub>r</sub> (%)	10.6	-	3.0	5.6	-	8.3	5.7	-
	S <sub>R</sub> (µg/kg)	3.43	-	2.63	3.91	-	5.42	2.56	-
	RSD <sub>R</sub> (%)	20.0	-	9.5	8.8	-	9.1	7.2	-
	<b>HORRAT</b>	<b>0.68</b>	-	<b>0.35</b>	<b>0.34</b>	-	<b>0.37</b>	<b>0.27</b>	-
AZA3	No. of labs a(b)	11(1) <sup>a</sup>	12	11(1) <sup>a</sup>	11(1) <sup>a</sup>	12	12(0)	11(1) <sup>a</sup>	12
	Mean concentration (µg/kg)	53.5	<LOQ	84.4	81.4	<LOD	186.0	108.9	<LOD
	S <sub>r</sub> (µg/kg)	1.99	-	2.88	3.16	-	3.58	3.77	-
	RSD <sub>r</sub> (%)	3.7	-	3.4	3.9	-	1.9	3.5	-
	S <sub>R</sub> (µg/kg)	3.39	-	4.80	5.30	-	18.82	7.71	-
	RSD <sub>R</sub> (%)	6.3	-	5.7	6.5	-	10.1	7.1	-
	<b>HORRAT</b>	<b>0.25</b>	-	<b>0.25</b>	<b>0.28</b>	-	<b>0.49</b>	<b>0.32</b>	-
AZA group total toxicity (µg AZA1 eq./kg)	No. of labs a(b)	11(0)	11	11(0)	11(0)	10	10(0)	10(0)	10
	Mean concentration (µg AZA1 eq./kg)	175.0	<LOQ	280.1	385.2	<LOD	615.2	350.8	<LOD
	S <sub>r</sub> (µg AZA1 eq./kg)	7.72	-	5.30	11.48	-	15.47	7.94	-
	RSD <sub>r</sub> (%)	4.4	-	1.9	3.0	-	2.5	2.3	-
	S <sub>R</sub> (µg AZA1 eq./kg)	21.47	-	25.34	39.16	-	54.81	38.10	-
	RSD <sub>R</sub> (%)	12.3	-	9.0	10.2	-	8.9	10.9	-
	<b>HORRAT</b>	<b>0.59</b>	-	<b>0.47</b>	<b>0.55</b>	-	<b>0.52</b>	<b>0.58</b>	-

a(b) = "a" is number of labs remaining after removal of number of outliers indicated by (b); S<sub>r</sub> standard deviation of the repeatability; S<sub>R</sub> standard deviation of the reproducibility; <sup>a</sup>) lab 19 outlier single Grubbs test

Table 6: Method performance for PTX2 and YTX-group toxins in mussels, oysters and cockle, quantified against PTX2 and YTX respectively

Shellfish matrix		Materials							
		1 Mussel	2 Mussel (+PTX2 at 97 µg/kg)	3 Mussel	4 Oyster	5 Mussel	6 Mussel	7 Cockle	8 Oyster (+PTX2 at 202 µg/kg)
PTX2	No. of labs a(b)	10	10(0)	10	10	10(0)	11	9(2) <sup>a</sup>	11(0)
	Mean concentration (µg/kg)	<LOD	82.3	<LOD	<LOD	10.1	<LOD	15.2	186.3
	S <sub>r</sub> (µg/kg)	-	Not available	-	-	0.87	-	1.12	7.92
	RSD <sub>r</sub> (%)	-	Not available	-	-	8.7	-	7.4	4.3
	S <sub>R</sub> (µg/kg)	-	10.98	-	-	3.13	-	1.47	38.38
	RSD <sub>R</sub> (%)	-	13.3	-	-	31.0	-	9.7	20.6
	Recovery	-	85	-	-	-	-	-	92
	HORRAT	-	<b>0.44</b>	-	-	<b>0.97</b>	-	<b>0.32</b>	<b>1.00</b>
YTX	No. of labs a(b)	9(1) <sup>b</sup>	9(1) <sup>b</sup>	9(1) <sup>b</sup>	10	11(0)	10(1) <sup>b</sup>	9(2) <sup>bc</sup>	11(0)
	Mean concentration (µg/kg)	143.9	1023.2	713.2	<LOD	290.6	206.9	172.7	39.1 <sup>d</sup>
	S <sub>r</sub> (µg/kg)	18.01	55.58	45.03	-	32.39	25.51	21.44	2.43
	RSD <sub>r</sub> (%)	12.5	5.4	6.3	-	11.1	12.3	12.4	6.2
	S <sub>R</sub> (µg/kg)	17.99	131.47	59.05	-	48.93	27.84	17.15	18.95
	RSD <sub>R</sub> (%)	12.5	12.8	8.3	-	16.8	13.5	9.9	48.4
	HORRAT	<b>0.58</b>	<b>0.81</b>	<b>0.49</b>	-	<b>0.87</b>	<b>0.66</b>	<b>0.48</b>	<b>1.86<sup>d</sup></b>
	45-OH-YTX	No. of labs a(b)	10(0)	10(0)	9(1) <sup>e</sup>	11	11(0)	11(0)	11(0)
Mean concentration (µg/kg)		98.6	651.6	418.2	<LOD	177.7	114.9	108.6	<LOQ
S <sub>r</sub> (µg/kg)		7.92	22.43	30.23	-	27.26	20.75	21.43	-
RSD <sub>r</sub> (%)		8.0	3.4	7.2	-	15.3	18.1	19.7	-
S <sub>R</sub> (µg/kg)		32.06	260.88	114.00	-	60.19	42.78	43.87	-
RSD <sub>R</sub> (%)		32.5	40.0	27.3	-	33.9	37.2	40.4	-
HORRAT		<b>1.43</b>	<b>2.35</b>	<b>1.49</b>	-	<b>1.63</b>	<b>1.68</b>	<b>1.81</b>	-
YTX group total toxicity (µg YTX eq./kg)		No. of labs a(b)	9(0)	9(0)	8(1) <sup>b</sup>	9	10(0)	9(1) <sup>b</sup>	8(2) <sup>bc</sup>
	Mean concentration (µg YTX eq./kg)	254.7	1702.2	1109.9	<LOD	462.2	310.4	380.8	<LOQ
	S <sub>r</sub> (µg YTX eq./kg)	16.06	60.10	70.65	-	56.70	34.47	43.94	-
	RSD <sub>r</sub> (%)	6.3	3.5	6.4	-	12.3	11.1	15.7	-
	S <sub>R</sub> (µg YTX eq./kg)	63.03	434.51	89.17	-	100.57	50.85	41.33	-
	RSD <sub>R</sub> (%)	24.7	25.5	8.0	-	21.8	16.6	14.7	-
	HORRAT	<b>1.26</b>	<b>1.73</b>	<b>0.51</b>	-	<b>1.21</b>	<b>0.86</b>	<b>0.76</b>	-

a(b) = "a" is number of labs remaining after removal of number of outliers indicated by (b); S<sub>r</sub> standard deviation of the repeatability; S<sub>R</sub> standard deviation of the reproducibility; <sup>a</sup>) labs 1 and 19 outliers in paired Grubbs test; <sup>b</sup>) lab 11 outlier in single Grubbs test; <sup>c</sup>) lab 19 outlier in paired Grubbs test; <sup>d</sup>) concentration is below LOQ (for LOD / LOQ different toxins see table 2); <sup>e</sup>) lab 2 outlier in paired Grubbs test

Table 7: Results for individual toxins to assess trueness based on quantification against the individual corresponding toxin calibrants

Material	Materials				
	Spiked homogenate I	Spiked homogenate II	FDMT1 material	CRM-MUS-B	
OA	No of labs a(b)	11(0)	11(0)	11(0)	9(1) <sup>a</sup>
	Mean concentration (µg/kg)	82.4	73.9	257.2	9507.1
	Target concentration (µg/kg)	100.0	91.0	275	10100
	S <sub>R</sub> (µg/kg)	20.5	17.6	35.8	1179.2
	RSD <sub>R</sub> (%)	25.4	23.8	13.9	12.4
	<b>Recovery (%)</b>	<b>80</b>	<b>81</b>	<b>94</b>	<b>94</b>
	HORRAT	1.09	1.05	0.71	1.09
DTX1	No of labs a(b)			10(0)	9(0)
	Mean concentration (µg/kg)			125.2	1380.0
	Target concentration (µg/kg)			133	1300
	S <sub>R</sub> (µg/kg)			22.7	388.1
	RSD <sub>R</sub> (%)			18.2	28.1
	<b>Recovery (%)</b>			<b>94</b>	<b>106</b>
	HORRAT			0.83	1.84
DTX2	No of labs a(b)			11(0)	
	Mean concentration (µg/kg)			643.4	
	Target concentration (µg/kg)			595	
	S <sub>R</sub> (µg/kg)			139.7	
	RSD <sub>R</sub> (%)			21.7	
	<b>Recovery (%)</b>			<b>108</b>	
	HORRAT			1.27	
AZA1	No of labs a(b)	10(0)		10(0)	
	Mean concentration (µg/kg)	39.4		615.4	
	Target concentration (µg/kg)	48		712	
	S <sub>R</sub> (µg/kg)	8.2		75.2	
	RSD <sub>R</sub> (%)	20.8		12.2	
	<b>Recovery (%)</b>	<b>82</b>		<b>86</b>	
	HORRAT	0.80		0.71	
AZA2	No of labs a(b)			12(0)	
	Mean concentration (µg/kg)			165.0	
	Target concentration (µg/kg)			198	
	S <sub>R</sub> (µg/kg)			14.7	
	RSD <sub>R</sub> (%)			8.9	
	<b>Recovery (%)</b>			<b>83</b>	
	HORRAT			0.42	

Material		Materials			
		Spiked homogenate I	Spiked homogenate II	FDMT1 material	CRM-MUS-B
AZA3	No of labs a(b)			12(0)	
	Mean concentration ( $\mu\text{g}/\text{kg}$ )			171.9	
	Target concentration ( $\mu\text{g}/\text{kg}$ )			186	
	$S_R$ ( $\mu\text{g}/\text{kg}$ )			25.3	
	RSD <sub>R</sub> (%)			14.7	
	<b>Recovery (%)</b>			<b>92</b>	
	HORRAT			0.70	
PTX2	No of labs a(b)	11(0)	11(0)	11(0)	
	Mean concentration ( $\mu\text{g}/\text{kg}$ )	186.3	74.6	73.5	
	Target concentration ( $\mu\text{g}/\text{kg}$ )	202	95	119	
	$S_R$ ( $\mu\text{g}/\text{kg}$ )	38.3	12.9	19.2	
	RSD <sub>R</sub> (%)	20.6	17.2	26.1	
	<b>Recovery (%)</b>	<b>92</b>	<b>79</b>	<b>62</b>	
	HORRAT	1.00	0.73	1.10	
YTX	No of labs a(b)	11(0)		11(1) <sup>b</sup>	
	Mean concentration ( $\mu\text{g}/\text{kg}$ )	267.1		371.2	
	Target concentration ( $\mu\text{g}/\text{kg}$ )	300		450	
	$S_R$ ( $\mu\text{g}/\text{kg}$ )	83.3		67.9	
	RSD <sub>R</sub> (%)	31.2		18.3	
	<b>Recovery (%)</b>	<b>89</b>		<b>82</b>	
	HORRAT	1.60		0.98	

a(b) = "a" is number of labs remaining after removal of number of outliers indicated by (b); a) lab 21 outlier single Grubbs test; b) lab 11 outlier single Grubbs test

Table 8: Comparison of toxin quantification against corresponding calibrant and close analogue calibrant

Toxin	Quantified against	materials								
		1 Mussel	2 Mussel	3 Mussel	4 Oyster	5 Mussel	6 Mussel	7 Cockle	8 Oyster	FDMT1
DTX1	OA ( $\mu\text{g}/\text{kg}$ )	29.5	64.9			58.5		85.4	13.5	115.1
	HORRAT	1.02	2.04			1.25		1.34	0.94	1.41
	DTX1 ( $\mu\text{g}/\text{kg}$ )	36.8	69.6			70.7		106.5	17.3	125.2
	HORRAT	0.63	0.53			0.50		0.61	0.88	0.83
DTX2	OA ( $\mu\text{g}/\text{kg}$ )	133.1		75.6	172.9		14.2	171.6	103.9	765.1
	HORRAT	0.62		0.91	1.01		1.06	0.80	0.92	0.82
	DTX1 ( $\mu\text{g}/\text{kg}$ )	111.0		63.5	142.0		13.5	144.1	87.7	643.4
	HORRAT	0.77		0.78	0.78		1.19	0.65	0.68	1.27
DTX1 + esters	OA ( $\mu\text{g}/\text{kg}$ )	36.4	66.6			97.7		132.5	23.9	110.3
	HORRAT	0.77	0.92			1.22		1.59	0.87	1.71
	DTX1 ( $\mu\text{g}/\text{kg}$ )	55.3	98.2			137.9		188.4	33.7	143.6
	HORRAT	1.00	1.04			0.90		0.87	0.80	0.91
DTX2 + esters	OA ( $\mu\text{g}/\text{kg}$ )	189.6	10.3 <sup>a</sup>	107.1	260.5		15.7	261.6	196.2	935.8
	HORRAT	1.78	2.37	1.57	2.05		1.52	1.52	2.07	1.78
	DTX2 ( $\mu\text{g}/\text{kg}$ )	182.1	9.4 <sup>a</sup>	105.6	251.5		13.5	233.3	160.0	933.9
	HORRAT	0.98	2.04	1.21	1.42		1.62	1.47	1.29	1.07
AZA2	AZA1 ( $\mu\text{g}/\text{kg}$ )	16.0		24.8	40.4		56.6	32.1		150.9
	HORRAT	0.37		0.37	0.48		0.58	0.48		0.81
	AZA2 ( $\mu\text{g}/\text{kg}$ )	17.2		27.5	44.6		59.8	35.5		165.0
	HORRAT	0.68		0.35	0.34		0.37	0.27		0.42
AZA3	AZA1 ( $\mu\text{g}/\text{kg}$ )	37.3		55.9	54.4		126.7	72.6		113.4
	HORRAT	0.75		0.74	0.85		0.71	0.72		0.82
	AZA3 ( $\mu\text{g}/\text{kg}$ )	53.5		84.4	81.4		186.0	108.9		171.9
	HORRAT	0.25		0.25	0.28		0.49	0.32		0.70

<sup>a</sup> concentration is at LOQ (for LOD / LOQ different toxins see table 2)