Cefas contract report C3011

In-house validation of an LC-MS/MS method for the determination of lipophilic toxins in shellfish species typically tested in the United Kingdom

Contract Reference: FS235004 (P01005)

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(Cefas contract C3011)

Final report (March 2011)

Executive summary.

In January 2011, Commission Regulation Number 15/2011 stated that a liquid chromatography-mass spectrometric (LC-MS/MS) method should be applied as the reference method for the measurement of marine lipophilic toxins (MLTs) in shellfish, thus replacing the live animal assay. The new regulation will apply as from 1 July 2011 and to allow Member States to adapt their methods to LC-MS/MS; the mouse bioassay may still be used until 31 December 2014. An in-house method validation scheme involving High Performance Liquid Chromatography tandem mass spectrometry (HPLC-MS/MS) was undertaken to establish method performance characteristics for the detection and quantitation of twelve regulated toxins in shellfish species commonly tested in United Kingdom's statutory MLT monitoring programmes. Shellfish species included mussel, cockle, oysters, scallops and clams.

A published liquid chromatographic method using an alkaline gradient was selected and refined to separate the MLTs within 22.5 min. A mass spectrometric method was developed involving electrospray ionisation and multiple reaction monitoring of two transition ions per toxin. To isolate MLTs from shellfish tissues, the European Union's Reference Laboratory for Marine Biotoxins (EU-RL) extraction procedure was applied.

The HPLC-MS/MS method demonstrated satisfactory selectivity. No visible interfering chromatographic peaks were evident at or close to expected retention times of the target analytes. Linearity of response was acceptable for the majority of toxins including okadaic acid/dinophysistoxins 1 and 2 (OA/DTX1/2), pectenotoxins 2 and 11 (PTX2/11), azaspiracids 1, 2 and 3 (AZA1/2/3) and non-regulated 13-desmethyl spirolide C (SPX1) and gymnodimine (GYM) prepared as solvent-based and shellfish matrix matched calibration standards. A linear range equivalent of 10-150% of the regulatory limit (RL) was evident for yessotoxin (YTX), whereas linear ranges for other toxins were <10 to 200% RL.

Method limits of detection (LOD) for OA/DTXs were 7-16 µg/kg. Limits of quantitation (LOQ) ranged from 23-58 µg/kg. On a toxic equivalence basis, LOQs represented 52-76% of the 160 µg[OA eq.]/kg limit. The sensitivity of the method was seen to be limiting for this toxin group. Respectively, LOQs for PTX2 and AZA1 were good and ≤10 and ≤5 µg/kg; for YTX, LOQs were between 28 and 105 µg/kg. Method recoveries were determined from shellfish tissues fortified with OA at 38% RL, DTX1/2, PTX2 and AZA1 at 63% RL, and YTX at 25% RL. An average recovery of 93% was calculated for these toxins extracted from all tissues of interest. Lower recoveries exhibited by PTX2 (82-97%) and AZA1 (80-92%) may have been influenced by co-extracted matrix suppression effects. A recovery range of 71 to 671% (YTX/Razor clam) was observed after spiking tissues with analytes close to method LOQs. Severe enhancement effects exerted on YTX would have most likely biased recoveries of this toxin.

For tissues spiked at the higher concentrations listed above, mean method repeatability relative standard deviations (RSDs) were considered acceptable. For OA/DTXs, these were 7-9%, <5% for PTX2 and AZA1, and ~10% for YTX. Higher RSD values were seen for YTX determinations from oysters, Queen scallop and Razor clam species. For the determination of MLTs at concentrations close to LOQs, RSDs were higher for most toxin/shellfish combinations. A majority of within-laboratory method reproducibility RSDs were also acceptable and <11%. However, higher RSDs (range 9-21%; average 15%) were apparent for YTX measurements.

Method ruggedness studies were undertaken to assess the stability of the method. Outcomes from statistical tests inferred that none of the parameter changes made to the

method had significant effects on its stability. The method was considered rugged for determination of OA/DTXs, PTX2, AZA1 and YTX in shellfish tissues of interest.

Preliminary standardised and expanded measurement uncertainties for OA/DTXs, PTX2/11, AZA1/2/3 and YTX/45 OH YTX were calculated from results of the validation exercises. Combined standardised uncertainties ranged from 0.05 to 0.40 and mean values for each shellfish species were 0.10 to 0.26. Expanded uncertainties (k=2) ranged from 0.09 (AZA1/King scallop) to 0.80 (YTX/mussel).

The HPLC-MS/MS method was transferred to a newly acquired instrument deploying Ultra Performance Liquid Chromatography (UPLC™) and 'fast' MS data acquisition. After method conversion to 'fast' LC, sample analysis time was reduced by 65% (to <8 min). This improvement ensures the UPLC-MS/MS method is responsive to overnight processing of up to 40 samples and has the capacity to meet the demands of statutory monitoring programmes.

The UPLC-MS/MS method demonstrated acceptable linear relationships between concentration and detector response. The working linear range was equivalent to 4-400% RLs for OA, DTX1/2, PTX2 and AZA1 toxins. For YTX, linear range extended to four times the RL and an improvement over the performance of the conventional HPLC-MS/MS method was seen. For all shellfish matrices, instrumental LOQs for OA/DTXs were estimated to be <10 µg/kg. On a toxic equivalence basis, summed average LOQs were 12% of 160 µg[OA eq.]/kg. Compared to the HPLC-MS/MS method, improvements in method sensitivity were gained from the application of UPLC-MS/MS to this group of toxins. For PTX2 and PTX11, LOQs were similar and <3 µg/kg; LOQs for YTX indicated that lower concentrations can be achieved by UPLC-MS/MS. Satisfactory statistical comparability was shown between UPLC- and HPLC-MS/MS for the quantitation of most regulated toxins [free OA/DTXs, total OA/DTX1 (after hydrolysis), PTX1/2 and AZA1/2/3] from naturally contaminated mussel, cockle and Pacific oyster tissues.

Throughout the in-house validation period, the HPLC-MS/MS method was applied to test samples provided in proficiency exercises organised by the programme, *Quality Assurance of Information for Marine Environmental Monitoring in Europe* (QUASIMEME). Derived *z*-scores showed the method performed satisfactorily for a majority of measurements of free and total (hydrolysed) OA and DTXs, and AZA1/2/3 and in a range of shellfish matrices. The HPLC-MS/MS method was also applied in three interlaboratory studies organised by the Federal Office of Consumer Protection and Food Safety (BVL; Germany), the Institute of Food Safety (RIKILT; The Netherlands) and the EU-RL (Spain).

In the BVL study involving the determination of OA/DTXs, PTX2, AZAs and YTX toxins in cooked mussel tissues, and oyster and clam extracts, *z*-scores of ≤|2| indicated the method performed satisfactorily and acceptable recoveries (94-106%) were achieved from spiked extracts. The HPLC-MS/MS method successfully achieved quality control criteria (correlation coefficient $r^2 \geq 0.98$; response drift ≤25%) for all toxin (OA, DTX1/2, PTX2, AZA1/2/3 and YTX) calibrations.

In the RIKILT study, participants were required to apply the alkaline LC gradient as adopted for the in-house validation scheme reported herein. A mussel matrix match standard approach was also applied to toxin calibration and quantitation. The method met quality control criteria (correlation coefficient $r^2 \geq 0.98$; response drift ≤25%) for most toxin calibrations with the exception of total OA and DTX2 calibrations. It was found that the laboratory mean concentrations of free OA were consistently higher than assigned mean values by a factor of ~1.2. By direct quantitation using DTX1 and DTX2 calibrations, free and total DTX1 and DTX2 levels were similar to assigned means. Recoveries of PTX2 from spiked tissues were ≥90%, and laboratory and assigned means were similar. Little difference was found between laboratory and assigned AZA2 means when this toxin was determined directly, using AZA2 or indirectly using AZA1 calibrations. However,

quantitation of AZA3 concentrations by direct quantitation resulted in laboratory means being higher than assigned values by a factor of ~1.5.

Valuable information was gained on the performance of the HPLC-MS/MS method from our participation in these interlaboratory studies. The application of mussel matrix correction was found to have the potential to improve the quality of our toxin data derived from the analysis of a range of shellfish species including mussel, cockle, oyster and clams. From the in-house validation exercises, it was evident that the HPLC-MS/MS method was prone to complex matrix influences. These ranged from negligible effects to strong enhancement/suppression effects. Diluting-out matrix influences appeared to reduce effects on certain toxin responses but had little or no impact on effects exerted on other analytes. As a method for moderating matrix interferences, extract dilution was regarded as an impractical approach. Extract clean up by solid phase extraction did appear to overcome mussel and oyster matrix enhancement effects on YTX responses. However, the variable performance of the method made this approach less efficient in reducing matrix influences on other toxins.

From the technical perspective, the HPLC-MS/MS method is convenient, practical and fit-for-purpose. We recommend that it should be implemented in the UK statutory lipophilic toxin monitoring programmes and applied to the measurement and reporting of MLTs in official control samples of those shellfish species targeted for method validation. Following implementation, we would recommend that additional work is undertaken to further refine and optimise the analytical method. Further research would include: the need for, and effectiveness of the use of matrix matched standards (arising from findings from interlaboratory studies); the incorporation of new lipophilic toxin reference standards (*e.g.,* AZA2, AZA3) into methodologies as they become commercially available; and validation performance checks and verifications for minor species to allow the method to be extended to all national official control shellfish samples.

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1. Introduction.

1.1. Marine lipophilic toxins, European regulation and methods of detection.

Marine phycotoxins are naturally occurring, secondary metabolites produced by certain phytoplanktonic species such as the *Dinophysis* and *Prorocentrum* genera. As a food source, via filter feeding mechanisms and without causing adverse effects to the shellfish itself, these harmful algae can bioaccumulate within tissues of bivalve molluscs. Depending on the quantities ingested by man, consumption of phycotoxin-contaminated shellfish may result in intoxication. Top predator wildlife including marine birds and mammals may also be affected (*e.g.*, Landsberg *et al*., 2009; Miller, 2009). Due to the presence of harmful phytoplankton in the vicinity of shellfish producing areas and the potential for uptake by shellfish, the economy of the seafood industry can also be impacted by restrictions being placed on harvesting and marketing (Hoagland *et al*., 2006). Thus, to safeguard the human consumer, regulation has been established and harmful phytoplankton and shellfish toxin monitoring programmes have been put in place, worldwide (Anon., 2004a; Anon., 2005). Based on physico-chemical properties, two classes of shellfish toxins exist and include hydrophilic and lipophilic compounds. To date, six groups of phycotoxins have been described as marine lipophilic toxins (MLTs). These are okadaic acid (OA) and its analogues, dinophysistoxins (DTXs), pectenotoxins (PTXs), azaspiracids (AZAs), yessotoxins (YTXs), cyclic imines and brevetoxins.

Toxins belonging to the OA group include DTX1 and DTX2 analogues. These are hydroxylated, polyether carboxylic acids (Appendix 1) and as the mode of action, inhibit serine and threonine phosphatases (PP1 and PP2A; Honkanen *et al.*, 1992). This group also includes OA, DTX1 or DTX2 esterified with saturated and unsaturated, long chain fatty acids. Collectively, these derivatives are termed as 'DTX3s'. The proportions of 'DTX3s' can reach as much as 100% of total OA group toxins (Villar-González *et al*., 2008). On consumption of shellfish contaminated with 'DTX3s', these acylated esterified forms can undergo hydrolysis within the human gut and convert to their parent forms, thus imposing toxic effects. Adverse effects such as nausea, vomiting, abdominal cramps, gastrointestinal disorder, and diarrhoea, (Garcia *et al*., 2005) may occur following ingestion of OA and/or DTX contaminated shellfish. Consequently, these toxins as well as azaspiracids are responsible for the syndrome known as diarrhetic shellfish poisoning (DSP) and commonly occur in shellfish produced in Europe. The current European Union's (EU) regulatory limit for the total amount of OA and DTXs is 160 µg[OA equivalents (eq.)]/kg whole shellfish tissue (Anon, 2004). Recently in their scientific opinion on this group, the European Food Safety Authority (EFSA 2008a) suggested that in order not to exceed the acute reference dose, the total OA/DTX content in shellfish tissue should be no more than 45 µg[OA eq.]/kg. For OA eq. concentrations to be determined, 'toxicity equivalent factors' (TEFs) for OA, DTX1 and DTX2 have been established (Table 1).

Under current EU legislation, pectenotoxins (PTXs) are also included within the OA group on the basis that they are produced by the same *Dinophysis* phytoplankton and can co-occur in shellfish tissues. These are polyether-lactone compounds (Appendix 1) and to-date, up to 15 analogues have been elucidated. In European shellfish, the most commonly found is PTX2 along with its metabolites, PTX2 seco acid and 7-*epi* PTX2 seco acid (Vale and Sampayo, 2002). Pectenotoxins do not share the same mode of action as the OA group and no human intoxications with DSP syndromes have been reported solely due to their presence in shellfish tissue. However, liver damage in mice (development of vacuoles and hepatocytes deformation; Espina and Rubiolo, 2008) and histopathological

changes in the liver and stomach of mice with no diarrhoeic effects (Miles *et al*., 2004) have been demonstrated. Recently, EFSA (2009) concluded that PTX1 and PTX2 should be separated from the OA group and their toxicity should be expressed on a PTX2 (eq.) basis. To determine toxicity due to PTXs, provisionally a TEF equal to 1 has been proposed for PTX1, PTX2, PTX3, PTX4, PTX6 and PTX11. Additionally, a limit of no more than 120 µg[PTX2 eq.]/kg has been indicated.

Table 1. European regulated marine lipophilic toxins and regulatory limits (Anon. 2004).

Toxin	Abbreviation	Toxicity equivalent factors (TEFs)	Regulatory limit
Okadaic acid	OA	1.0	160 µg[OA eq.]/kg(†)
Dinophysistoxin 1	DTX1	1.0	
Dinophysistoxin 2	DTX2	0.6	
Pectenotoxin 1	PTX1	1.0(‡)	
Pectenotoxin 2	PTX2	1.0(‡)	
Azaspiracid 1	AZA1	1.0	160 µg[AZA1 eq.]/kg
Azaspiracid 2	AZA2	1.8	
Azaspiracid 3	AZA3	1.4	
Yessotoxin	YTX	1.0	1 mg[YTX eq.]/kg
1a-*homo* yessotoxin	1a-*homo* YTX	1.0	
45 OH yessotoxin	45 OH YTX	1.0	
45 OH 1a-*homo* yessotoxin	45 OH 1a-*homo* YTX	0.5	

(†) includes PTX1 and PTX2. (‡) Proposed TEFs for PTX1 and PTX2 (after EFSA, 2009).

Azaspiracid (AZA) toxins are nitrogen-containing, polyether compounds (Food and Agriculture Organisation 2005) and consist of a spiral ring assembly containing a heterocyclic amine as well as an aliphatic carboxylic acid moiety (Appendix 1). Toxic effects experienced by the human consumer of AZA-contaminated shellfish are similar in nature to those demonstrated by OA and DTXs (James *et al*., 2002 and 2004). Over 20 analogues have been identified with AZA1, AZA2 and AZA3 being the most commonly found compounds in shellfish tissues (Rehmann *et al*., 2008). These toxins are wide in their geographical distribution and have been found in shellfish producing regions of Europe, North Africa, and North and South America (*e.g.,* Torgersen *et al*., 2008; Amzil *et al*., 2008; Vale *et al*., 2008a; Elgarch *et al*., 2008; Klontz *et al*., 2009; Lopez-Rivera *et al*., 2009). The current regulatory limit is 160 µg[AZA1 eq.]/kg and TEFs have been estimated for the three commonly occurring compounds (Anon, 2004; Table 1). The recent EFSA (2008c) review of AZA toxicity indicated a lower, safe value of 30 µg[AZA1 eq.]/kg.

Yessotoxins (YTXs) are also polyether compounds (Appendix 1) and composed of eleven, contiguously transfused ether rings, an unsaturated side chain and two sulphate esters. Approximately 90 YTX derivatives have been identified by Miles *et al*. (2005). The current regulatory limit for YTX and its analogues - 1a-*homo* YTX, 45 OH YTX and 45 OH 1a *homo* YTX is 1 mg[YTX eq.)/kg (Table 1). These analogues were considered by EFSA (2008b) as the most important and have been found in shellfish produced within European waters (*e.g.* Draisci *et al*., 1999; Aasen *et al*., 2005a; Vale *et al*., 2008b). However, no human intoxications due to the ingestion of YTX-contaminated shellfish have been reported. Experiments using mice have shown that via *intra*-peritoneal (*i.p.*) injection, YTX toxicity is high with a LD_{50} for mice of 750 µg/kg (Aune *et al*., 2002). Conversely, oral administration of ≤10 mg[YTX]/kg doses to mice only resulted in swelling of heart muscle cells. Recently, EFSA (2008b) concluded a limit of 3.75 mg[YTX eq.]/kg would provide consumer protection.

Spirolides [SPXs; 13-desmethyl spirolide C (SPX1); Appendix 1] including gymnodimines (GYMs) are members of the cyclic imine toxin group. Via mouse *i.p*. injection, these are classed as 'fast-acting' toxins as they can cause death within minutes (Richard *et al*., 2000). To date, cyclic imines have not been implicated in reported incidences of human intoxication. As with AZAs, SPXs demonstrate a global distribution (*e.g*., Aasen *et al*., 2005b; Alvarez *et al.*, 2010) whereas GYMs have been evident in shellfish from New Zealand (Seki *et al*., 1995). The lipid-soluble, brevetoxins (BTXs) and their metabolites are cyclic polyether compounds. They can cause the neurological shellfish poisoning (NSP) syndrome and effects include reduction of the respiratory rate, cardiac disturbances, cramps, diarrhoea, vomiting (Watkins *et al*., 2008). Reported intoxications due to BTXs seem to be confined to Florida and the Gulf of Mexico (Heil, 2009), and New Zealand (Ishida *et al*., 2004). Their presence in European shellfish has not yet been recorded. Currently, no EU legislation exists for cyclic imines or brevetoxins.

The official method for detecting OA, DTXs, PTXs, AZAs and YTXs in shellfish tissue is a qualitative, biological assay (Anon 2004) and involves a mouse bioassay (MBA). A rat bioassay may also be deployed for the qualitative detection of OA/DTX and AZA toxins. Within Europe, a standardised procedure has been published by the European Reference Laboratory on Marine Biotoxins (EU-RL, 2009) for performing the MBA. Extracts are prepared to isolate lipophilic toxins from the whole shellfish tissue or from the hepatopancreas, and these extracts are injected into three, 20 g mice. A sample containing MLTs is reported as positive for the presence of lipophilic toxins when at least two of the animals die within 24 hours following injection (as the methodical endpoint; Anon., 2005). Besides the MBA's responses to MLTs that regularly appear within European shellfish and at concentrations approximating to the regulated limits, the assay can also provide an alert to the presence of other, bioactive compounds. However, the limitations of the MBA outweigh its benefits. The assay's inability to provide sensitive and quantitative information on specific toxin concentrations and compositions restrict its use. Its susceptibility to interfering, co-extracted free fatty acids can also lead to false positive results (Lacaze *et al*., 2007). Additionally, there is a legal and ethical obligation to promote the development of alternative techniques for the reduction and replacement of the use of animals in testing procedures and scientific or experimental research (Anon., 1986).

Commission Regulation (EC) No. 2074/2005 (Anon., 2005) stated that analytical methods such as liquid chromatography-mass spectrometry (LC-MS/MS), *in vitro* (*e.g.,* functional assays) and biochemical methods (*e.g.*, enzyme-linked immunosorbent assays) shall be used as alternatives or supplementary to the MBA provided that:

- they provide an equivalent (to the MBA) level of public health protection
- they can, either alone or combined, detect those toxins listed in Table 1
- total toxicity shall be established using conversion factors (TEFs) based on the toxicity information available for each toxin, and
- the performance of these methods shall be defined following validation performed to internationally agreed protocols

In January 2011, Commission Regulation (EC) No. 15/2011 (Anon., 2011), an amendment of Regulation No. 2074/2005, stated that the MBA is to be replaced and an LC-MS/MS method is to be the reference method for the detection of lipophilic toxins. Under the co-ordination of the EU-RL, the analytical method has been validated at the interlaboratory level involving European Member State participation (EU-RL, 2009) and method performance criteria have been established. The method should be applied as a matter of routine, both for the purposes of official controls at any stage of the food chain

and own-checks by food business operators. Methods other than LC-MS/MS can be applied provided they fulfil the EU-RL method performance criteria, and that they have been validated at the single laboratory level and have been successfully tested via recognised proficiency schemes. However, should results be challenged, the reference method shall be the EU-RL method. The new regulation will apply as from 1 July 2011 and the MBA may still be used until 31 December 2014. This is to allow Member States to adapt their methods to LC-MS/MS. After this date, the MBA may be used only during the periodic monitoring of shellfish production areas for detecting new or unknown MLTs.

The advantages of LC-MS/MS are sensitivity, precision, automation, quantitation and confirmation of toxin identity. Most MLTs are well suited to LC separation due to their lability, polarity and non-volatility. Where LC-MS/MS carries a high operating cost, *in vitro* functional assays or biochemical assays are ideal for 'screening out' negative samples. These assays are low in cost, provide rapid sample throughput and the detection of new bioactive compounds. However, in the presence of co-extracted shellfish matrix interferences, false positive *and* false negative results can occur (*e.g.*, Vale *et al*., 2009). Specific and accurate quantitation cannot be achieved with immunoassays where shellfish samples contain groups of toxins and where variable levels of individual toxins exist. In most instances, such techniques require a level of confirmation by more quantitative approaches and comprehensive validation exercises to describe method performance characteristics.

To chromatographically separate MLTs, LC methods using C_8 or C_{18} reversed phase columns and either acidic gradients (pH ~3; Quilliam *et al*. 2001; McNabb *et al*., 2005; Fux *et al*. 2007) or a neutral (pH 6.8) gradient (Stobbo *et al*., 2005) have been described. Acceptable chromatographic performance can be attained for the regulated MLTs, although overlapping elution of several compounds do occur especially where an acidic gradient is applied. Where a toxin efficiently ionises in one particular ionisation mode and closely elutes with a compound amenable to ionisation in the opposite mode, separation is effected by mass differences and by the MS instrument rapidly switching between ionisation modes and collecting data. However, where an instrument is not capable of such rapid polarity switching, sensitivity of detection can be compromised and two analytical runs may be required to analyse a single extract containing a mixture of MLT groups. More recently, an alkaline (pH 11) LC gradient was reported by Gerssen *et al*. (2009). Within one analytical run, MS data for OA, DTXs and YTXs were acquired as a cluster and separately to PTXs, AZAs, and SPXs. This excludes the need for rapid, polarity switching of the MS instrument or multiple analytical runs. Chromatographic peaks were observed to be narrow and compounds such as YTXs, were reported to be stable under alkaline conditions.

In-house validation of an LC–MS/MS method has been published by McNabb *et al*. (2005). An inter-laboratory study involving eight laboratories showed within-laboratories repeatability (RSD_r) from 8-12% for OA, DTX1, DTX2, AZA1, and YTX, between-laboratories reproducibility (RSD_R) were from good to adequate, and HorRat values ranged from 0.8-2.0. With the advent of modern LC technology involving ultra pressure liquid chromatography (UPLC™) coupled to fast data acquisition MS instrumentation, a 'fast' multi-MLT method has been reported by Fux *et al*. (2007). With this method, all EU-regulated toxins including some of their analogues were capable of being detected in less than seven minutes. The method published by Gerssen *et al*. (2010) has been validated at the single laboratory level. International collaborative studies co-ordinated by the German and the Netherlands National Reference Laboratories for marine biotoxins as well as the EU-RL were conducted throughout 2010. For laboratories involved in developing or

validating LC-MS/MS methods with the aim of replacing animal assays, these studies have provided opportunities for method validation at the international level.

1.2. Aim and objectives of the validation scheme.

The aim of the work reported herein was to validate a High Performance Liquid Chromatography with tandem mass spectrometric (HPLC-MS/MS) method and describe its performance characteristics for the determination of EU-regulated MLTs in shellfish commonly produced in United Kingdom (UK) waters and which are tested by the MBA. The specific objectives of the validation scheme were:

- to adopt an appropriate extraction procedure for the isolation of the regulated MLTs from a range of shellfish tissues including Common mussel, Common cockle, Pacific and Native oysters, King and Queen scallops and Hard and Razor clams
- to select and where necessary, refine an LC method suitable for toxin separation, and develop an MS/MS detection method for toxin quantitation in extracts from such shellfish
- under an agreement with the Institute for Marine Biosciences, National Research Council Canada:
  - to procure non-commercially available MLT reference materials (RMs; *i.e.*, pre-certified standards DTX1, DTX2, AZA2 and AZA3 and non-certified PTX1 and PTX11 *plus* a MLT-contaminated mussel tissue RM) to supplement stocks of commercially-available standards (OA, PTX2, AZA1 and YTX)
  - this was to perform optimisation for LC separation and MS/MS detection of most of the regulated as well as two, non-regulated toxins - SPX1 and GYM
- to undertake a single laboratory validation (SLV) scheme to establish the performance characteristics of the selected extraction and analytical methods. This was to be achieved by applying, as closely as possible, harmonised SLV guidelines set out by the International Union of Pure and Applied Chemistry (Thompson *et al.,* 2002) and, where quantities permitted, utilising the acquired RMs in a series of method validation exercises. The following performance characteristics were to be evaluated and described: selectivity; instrument calibration; linearity and working linear range; method limits of detection and quantitation; extraction recovery efficiencies; within-laboratory method repeatability and reproducibility; influence of co-extracted shellfish matrix on toxin measurement; method ruggedness; and measurement uncertainty
- on completion of the SLV scheme:
  - to transfer and convert the HPLC-MS/MS method to a second, more modern instrument involving Ultra Pressure LC separation and 'fast' MS data acquisition (UPLC-MS/MS)
  - using the newer instrument, to verify a limited number of the performance characteristics (selectivity, linearity and working linear range, and limits of sensitivity) established from the SLV exercises involving HPLC-MS/MS
  - to demonstrate that the results obtained by both instruments were comparable
- to bench mark and report on the performance of the optimised and characterised HPLC-MS/MS method through participation in interlaboratory studies such as proficiency testing schemes and collaborative trials.

With the primary aim of producing results of acceptable quality which meet the level of confidence required in shellfish safety decision making processes, the purpose of characterising the analytical method was to provide a potential method replacement to the animal assay for monitoring and quantifying MLTs in UK bivalve molluscs.

2. Materials, methods and pre-validation studies.

2.1. Lipophilic toxin standard solutions and preparation of reference standard solutions.

Individual marine lipophilic toxin (MLT) reference standards dissolved in methanol (MeOH) were purchased from the Institute for Marine Biosciences (IMB, National Research Council Canada, Halifax, Nova Scotia, Canada). These are listed in Table 2. Stock solutions of individual toxins were prepared by quantitatively transferring materials from sealed ampoules to 10 mL series A volumetric flasks and making-up to volume with MeOH. Solutions were then stored at <-20°C prior to use and for up to 12 months. Using calibrated pipettes, aliquots of these solutions were transferred to 5 mL series A flasks to prepare mixtures of calibration standard solutions at different concentrations for the purpose of calibration, quantitation or to fortify shellfish tissues for validation exercises.

Table 2. List of certified, non-certified reference standard solutions and MLT-contaminated extracts used in pre-validation studies and single laboratory validation exercises. (Non-regulated toxins SPX1 and GYM are highlighted).

Toxin	Acronym	Description
Okadaic acid	OA	14.3 µg/mL; certified reference standard
Dinophysistoxin 1	DTX1	16.1 µg/mL; pre-certified reference standard
Dinophysistoxin 2	DTX2	8.2 µg/mL; pre-certified reference standard
Pectenotoxin 1	PTX1	~1 µg/mL; non-certified reference standard
Pectenotoxin 2	PTX2	8.6 µg/mL; certified reference standard
Pectenotoxin 2 *seco acid*	PTX2*sa*	Non-certified, reference standard
Pectenotoxin 11	PTX11	8.8 µg/mL; non-certified reference standard
Azaspiracid 1	AZA1	1.24 µg/mL; certified reference standard
Azaspiracid 1, 2, 3	AZA1 AZA2 AZA3	AZA1 0.21 µg/mL;AZA2 0.14 µg/mL; AZA3 0.094 µg/mL pre-certified reference standard mixture
Yessotoxin	YTX	5.3 µg/mL; certified reference standard
1a-*homo* YTX, **45 hydroxy YTX,** **45 hydroxy 1a-*homo* YTX**	*homo* YTX 45 OH YTX 45 OH *homo* YTX	Methanolic mussel extracts from Italy, Norway and New Zealand
13-desmethyl spirolide C	SPX1	7.0 µg/mL; certified reference standard
Gymnodimine	GYM	5 µg/mL; certified reference standard

2.2. Materials and reagents.

Acetonitrile, MeOH and water used for the preparation of MLT calibration standard solutions, spiking solutions, shellfish tissue extraction and mobile phases for liquid chromatographic (LC) separation were of High Performance Liquid Chromatography (HPLC) grade (Rathburns Chemical Co., Walkerburn, Scotland). Mobile phase modifiers including ammonium hydroxide and ammonium hydrogencarbonate were of analytical grade (Sigma-Aldrich, Poole, England). For LC separation, a retention time marker solution was prepared and included in batch analyses of sample extracts obtained from for each of the validation exercises. The extract was deployed to assure the chromatographic elution order of each of the target analytes. A blend of MLT-contaminated mussel homogenates was double extracted with 100% MeOH and this was filtered using a 0.2 µm nylon syringe filter. Additional toxins such as SPX1 and GYM were spiked into the extract to complete the suite of toxins. The solution was stored at -20 °C prior to use.

Prior to performing validation exercises, approximately 0.5 kg of whole shellfish tissues, selected to represent those species commonly tested in the United Kingdom (UK), were obtained from the English, Welsh and Scottish statutory toxin monitoring programmes. For each species, whole tissues were removed from the shell, pooled and homogenised by Ultra Turrax™. Homogenised tissues were stored in glass containers at

<-20°C. When required, these materials were completely thawed and re-homogenised prior to use and included the following shellfish species:

- Common mussel (*Mytilus edulis*)
- Common cockle (*Cerastoderma edule*)
- Pacific oyster (*Crassostrea gigas*)
- Native oyster (*Ostrea edulis*)
- King scallop (*Pecten maximus*)
- Queen scallop (*Aequipecten opercularis*)
- Hard clam (*Mercenaria mercenaria*) and
- Razor clam (*Enis* spp.).

2.3. Analytical instrumentation.

For the single laboratory validation exercises, toxin separation was carried out using an *Agilent 1100* High Performance Liquid Chromatographic (HPLC) system (Agilent, Manchester, UK). This was equipped with a solvent reservoir, degasser module, binary pump, autosampler and temperature controlled column oven. Via an electrospray ionisation (ESI) interface, the LC was coupled to a *Quattro Micro* triple quadrupole mass spectrometer (MS/MS; Waters Ltd., Manchester, UK). The source and desolvation temperatures were set at 120 and 450°C, respectively. High purity (>99.99%) nitrogen was used for the cone (flow 100 L/h) and desolvation gasses (550 L/h). Argon (>99.999%) was used as the collision cell gas (default pressure; 4.5 to 5.0 x 10^{-6} Bar). The *MassLynx*^{TM} v.4.1 (Waters Ltd.) software was used for control of the LC gradient, source and collision cell parameters, data acquisition and processing.

2.4. Pre-validation studies.

Preliminary studies were undertaken prior to commencing the single laboratory validation (SLV) exercises involving:

- LC method selection and refinement for the separation of the target MLTs
- optimisation of the MS/MS instrumental parameters for the production of precursor and two transition ions for each MLT
- an evaluation of the efficiency of the adopted shellfish tissue extraction technique (European Union Reference Laboratory for marine biotoxins (EU-RL, 2006), and
- an assessment of the performance of the commonly applied hydrolysis procedure (as published by Mountford *et al*. 2001) in relation to the chemical reaction time required to convert acylated esters of OA, DTX1 and/or DTX2 (*i.e*., DTX3s) to their parent compounds for the measurement of total (free *plus* esterified) OA and DTXs.

2.4.1. Liquid chromatographic method selection and refinement.

As discussed in the Introduction, several LC-MS/MS methods applying acidic or neutral pH LC gradients have been reported for the LC separation of MLTs from shellfish tissues. Under acidic gradient conditions using C_8 or C_{18} columns, experience in the Cefas laboratory has shown that yessotoxins have a tendency to elute as broad peaks with poor symmetry with unstable retention times. Also, the non-regulated, 13-desmethyl spirolide C (SPX1) toxin is not retained. The neutral pH gradient described by Stobbo *et al*. (2005) has also been trialled in-house. Although two discrete chromatographic data acquisition windows could be achieved for positively and negatively ionisable toxins, broad and tailing peak shapes for AZAs and in particular, AZA2 (baseline width >1 min) were

apparent using C_8 and C_{18} columns. The alkaline (pH 11) LC gradient described by Gerssen *et al*. (2007; 2009) was reported to be equivalent or better in terms of performance of the more commonly applied 'acidic' separation methods. It is also capable of separating, as clusters, those negatively ionisable MLTs from toxins amenable to positive ionisation. This method was thus selected and adopted, trialled in-house and refined where necessary.

After initially trialling the alkaline LC gradient as published, a number of refinements were necessary to adapt it to the *Agilent 1100* LC and to the *Quattro Micro* MS interface used in this validation programme. Parameters such as different column dimensions, mobile phase percentage compositions and flow rates, gradient elution programmes, and column temperatures (25, 30, 35 and 40°C) were investigated to:

- improve the chromatographic resolution of OA and its isomer, DTX2
- attempt to chromatographically separate co-eluting PTX2 and SPX1, and
- improve the peak symmetry of AZA1, AZA2 and AZA3, and to reduce baseline widths to narrower (<1 min) dimensions.

2.4.2. Optimisation of MS parameters for the detection of the target toxins.

Following the refinement of the LC gradient, MS parameters were further refined and those regulated and non-regulated MLTs listed in Table 1 were 'tuned' to establish:

- a precursor (pseudo-molecular) ion of each target toxin, and
- two multiple reaction monitoring (MRM) transition (fragment) ions for the purposes of toxin quantitation and confirmation.

Using a 'T'-piece connector and an automatic syringe pump, solutions of individual MLTs (~0.1 ng/mL; 100% MeOH) were infused (at 10 µL/min) into the alkaline (pH11) mobile phase (0.29 µL/min) and set at 85% of the organic mobile phase. Optimised electrospray probe capillary voltages, MS source cone voltages and collision energies were then established for each toxin. Following this, the within-batch performance of the analytical method was assessed by multiple injections (10 µL*; n*=30; 12 h analytical run period) of the retention time marker solution (mussel extract) containing the suite of target MLTs (see 2.2, above).

2.4.3. Evaluation of the recovery efficiency of the adopted shellfish extraction method.

As a candidate extraction method to isolate analytes from MLT-contaminated shellfish tissues, the standard operating procedure (SOP) described by the European Union Reference Laboratory for Marine Biotoxins (EU-RL, 2006) was adopted. The method involves 2.0 ± 0.2 g of homogenised shellfish tissue being vortex mixed [3 minutes (min)] with 9.0 mL MeOH. The extract is centrifuged (2000 *g*; 20°C; ≥10 min) and the supernatant retained. The extracted shellfish pellet is further homogenised by Ultra Turrax™ (1 min) with another 9.0 mL MeOH, centrifuged and the supernatant combined with the first extract. The final extract volume is prepared to 20.0 mL by the addition of MeOH and the solvent-to-sample ratio (SSR) is 10:1.

With the objective of obtaining information on the performance of this approach and establishing the optimum number of methanolic extraction steps required to quantitatively recover maximum concentrations of targeted LTs, the EU-RL method was compared to single, triple and quadruple extraction steps. A naturally contaminated mussel tissue was used as the test matrix and three 2.0 g replicate samples were extracted for each

extraction procedure. Liquid chromatographic separation and mass detection was performed using the optimized methods described in sections 2.4.1 and 2.4.2, above. The content of OA, DTX1, DTX2, PTX2, AZA1, YTX and SPX1 in each extract was determined by external calibration using seven levels of calibration standards containing a mixture of these compounds. Recovered tissue concentrations of AZA2 and AZA3, and 45 OH YTX were determined indirectly by using calibration plots of AZA1 and YTX, respectively. The following extraction procedures were followed:

- a single step extraction was performed using vortex mixing only (3 min) and 18.0 mL 100% MeOH and centrifugation (2000 *g*; 20°C; 8 min). The final extract volume was made up to 20 mL (SSR 10:1)
- triple step extractions involved 2.0 g being initially vortex mixed (3 min) with 6.0 mL MeOH. This was followed by cenfiguation and then two homogenisation (Ultra Turrax^{TM}) steps with 2 x 6.0 mL MeOH (1 min each). All three extracts were combined to 20 mL (SSR 10:1), and
- quadruple step extractions were applied involving vortex mixing with 4.5 mL MeOH (3 min) as the first step followed by centifugation. After this, three homogenisation (Ultra Turrax^{TM}) procedures (3 x 4.5 mL MeOH; 3 min each) were performed. All extracts were combined (20 mL; SSR 10:1).

2.4.4. Performance of the chemical hydrolysis procedure to convert OA/DTX esters to their parent OA/DTX toxins.

The aim of this pre-validation exercise was to gain information on the performance of the hydrolysis method as described by Mountford *et al*. (2001). The procedure is commonly applied to crude, methanolic shellfish extracts to estimate total OA and DTX concentrations (*i.e*., free *plus* acylated ester forms) after exposure to hot (76°C), alkaline conditions for 40 min. Specifically, and for this exercise, hydrolysis reaction times were investigated in relation to the conversion and recovery of total OA, DTX1 and DTX2 concentrations. Assessments were made as to whether a degree of flexibility could be permitted for the duration of the reaction time. This is particularly pertinent for the application of this method to large batches of sample extracts requiring hydrolysis. Statistical Student *t*-tests were applied to the concentration data to identify differences of significance of concentration/reaction time combinations in comparison to total OA, DTX1 and DTX2 concentrations found after exactly 40 min of chemical reaction time.

Applying the EU-RL (2006) double extraction procedure (section 2.4.3), a blend of naturally contaminated Common mussel tissues was extracted to isolate free and esterified OA and DTX toxins. One mL extract aliquots (*n*=3 to 7) were transferred to 2 mL autosampler vials. The hydrolysis reaction was initiated by the addition of 125 µL 2.5 M sodium hydroxide to each vial, sealing with screw caps, vortexing mixing for 5 sec and by immediately placing all vials on a heater block (76±1°C). Three replicates were immediately neutralized at time 0 min (no heating) by the addition of 125 µL, 2.5 M hydrochloric acid. At 10 min, and then every five minutes after this up to 65 min, replicated extracts were removed and cooled to room temperature (21-25°C), and neutralized. Throughout the heating period, the security of the vial caps was periodically checked and maintained to minimise extract evaporation. Concentrations of total OA and DTX toxins in each hydrolysed extract were determined by the optimised HPLC-MS/MS method (sections 2.4.1 and 2.4.2), by random, sample injection analysis and by external calibration of OA, DTX1 and DTX2 calibrants prepared in MeOH.

2.5. Description of the methods applied in the single laboratory validation scheme.

Harmonised guidelines for SLV of analytical methods (Thompson *et al*., 2002) were applied as closely as possible for the validation of the combined extraction and analytical methods. As there is currently a lack of shellfish reference materials with certified quantities of MLTs, validation exercises were mostly performed using shellfish tissue homogenates fortified with mixtures of MLT standards as listed in Table 2. These standards included OA, DTX1, DTX2, PTX2, AZA1 and YTX. The non-regulated lipophilic toxins SPX1 and GYM were also incorporated into validation exercises.

2.5.1. Assessment of selectivity of the analytical method.

The selectivity of the LC-MS/MS method was assessed by qualitatively observing the presence or absence of interfering chromatographic peaks derived from endogenous compounds and which had been co-extracted from the toxin-free or 'blank' shellfish tissues. Such interferences may have the potential to co-elute with the target LT analytes and result in a signal response at the expected retention time of the analyte. Two gramme aliquots taken from toxin-free (blank; *n*=3) shellfish samples representing each of the eight shellfish species (Common mussel, Common cockle, Pacific and Native oysters, King and Queen scallops and Hard and Razor clams; section 2.2) were double extracted following the EU-RL (2006) standard operating procedure and analysed by the optimised LC-MS/MS method. To identify non-toxin, chromatographic interferences at the expected retention times of the target MLTs, qualitative examinations were then conducted of each MRM toxin transition chromatogram derived from the analysis of each representative shellfish species.

2.5.2. Calibration and linearity of detection of the *Quattro Micro* mass spectrometer.

The objective of these exercises was to:

- describe the performance of the *Quattro Micro* triple quadrupole mass spectrometer in terms of its range of linearity of detection for each of the following toxins, OA, DTX1, DTX2, PTX2, PTX11, YTX, AZA1, AZA2, AZA3, SPX1 and GYM prepared in 100% MeOH, and in shellfish extracts (matrix matched standards; MMS) with a solvent-to-sample ratio (SSR) of 10:1.

Linearity was evaluated for each of the eight test shellfish species. Eight levels of calibration solutions were prepared for most MLTs with the exception of YTX where six were prepared. Due to limited availability at the time of performing this exercise, only six levels were prepared for AZA2, AZA3 and PTX11 using non-certified reference standards. As an isomer of PTX1, PTX11 was used here to represent PTX1 since the former was unavailable in sufficient quantities and purity at the time of conducting this work. Aliquots of individual MLT stock solutions were transferred to 5 mL series A volumetric flasks to produce mixtures of toxin solutions. The range of concentrations of OA, DTX1, DTX2, PTX2 and AZA1 were equivalent to <10 to 200% of the regulatory limit (RL) of 160 µg[per toxin]/kg. The range of YTX concentrations were 10 to approximately 300% of the RL of 1 mg/kg. For the six series of calibration solutions for AZA2, AZA3 and PTX11 concentrations ranged from 14 to 290% of the RL for AZA2, 10 to 200% for AZA3 and 10 to 194% for PTX11.

For MeOH-based and shellfish MMS calibration solutions, n=7 repeated LC injections (10 µL) were performed randomly and for each level of concentration. Calibration curves ($y = mx + b$) represented by the plots of chromatographic peak area

response (*y*) for each toxin *versus* the concentration (*x*) of the calibration standards were constructed using a weighted (1/*x*) linear, least squares regression as the mathematical model. The linearity of the analytical method was evaluated visually through examination of calibration plots generated for individual toxins in each matrix. Correlation coefficients (r^2) were generated using all replicate data points and used in part, to identify any potential issues with calibration linearity. The assessment was extended with the analysis of the variance of the residuals into lack of fit and pure error. Specifically, an F-test "lack of fit" test was produced for each toxin in each matrix, where F was calculated using the equation described by Armitage and Berry (1994):

F = sum of squares (lack-of-fit) / degrees of freedom =
Sum of squares (pure-error) / degrees of freedom

$$= \frac{(\sum_{j=1}^{c}(n_j(\bar{Y}_j - \hat{Y})^2) / (c-2)}{(\sum_{j=1}^{c}\sum_{i=1}^{n_j}(Y_{ij} - \bar{Y})^2) / (n-c)}$$

Where: $\bar{Y}$ = mean y value at each concentration (X value)
$\hat{Y}$ = fitted y value from regression $\hat{Y} = mx + b$
n = total number of data points
c = total number of concentration points (X values)

So there are c distinct Xs, X_j (j = 1,2,...c) has n_i (i=1,2,...,n_j) observations of Y denoted by Y_{ij}.

The null hypothesis for the F-test was then rejected if F>F-critical at the appropriate degrees of freedom (1-α, c-2, n-c). Assumptions of the test include the normal distribution of y replicates, residuals and uniform variance of the residuals. Any regressions exhibiting lack of fit from this test were subsequently examined visually to demonstrate any significant non-linearity (AMC, 1994). If the residual pattern was found to support an interpretation of non-linearity, further clarification was sought through examination of potential experimental factors affecting the linearity and use of non-linear regression.

2.5.3. Investigations of co-extracted matrix influences on toxin signal response.

By calculating the ratio of calibration slopes derived from matrix matched standard (MMS) calibration plots to slopes derived from methanol-based calibrations, the influence of co-extracted shellfish matrix on toxin signal response was described. In addition and focussing on extracts from a selection of Common mussel and Pacific oyster samples, an extensive study was undertaken to evaluate the levels of shellfish matrix effects observed on the responses of lipophilic toxins. The objectives were to:

- describe the range of matrix influences on toxin signal responses derived from different mussel and oyster tissues acquired from different production areas in England, Wales and Scotland, and over a four month period (March to June 2010), and
- assess the effectiveness of (1) extract dilution and (2) extract clean up using solid phase extraction as potential methods for reducing or moderating these influences.

Utilising samples from national official control lipophilic toxin monitoring programmes, seven Common mussel and Pacific oyster samples were acquired from 14 shellfish producing areas (Table 3).

Table 3. Sampling dates and locations of Common mussel and Pacific oysters collected from official control marine toxin monitoring locations.

Common mussel sample no.	**Sampling date**	**Sample location**
1	17/03/2010	Swansea Bay South, Swansea, Wales
2	29/03/2010	Loch Roag – Linngeam, Cliatasay, Lewis and Harris, Scotland
3	27/04/2010	Seilebost, Lewis and Harris, Scotland
4	27/04/2010	Mawbray, Allerdale, England
5	17/05/2010	Bournemouth Pier, Poole, England
6	19/05/2010	Hamnavoe, Copister, Shetland Islands, Scotland
7	22/06/2010	Fishcombe Cove, Torbay, England
Pacific oyster sample no.	**Sampling date**	**Sample location**
1	17/03/2010	Pyefleet Spit, Colchester, England
2	30/03/2010	Loch Na Keal West, Eilean Casach, Argyll and Bute Council, Scotland
3	27/04/2010	Arisaig, Sgeirean Buidhe, Highland Council, Scotland
4	29/04/2010	West Bank, South Hampshire, England
5	17/05/2010	Seil Point, Ardencaple-Cyster, Argyll and Bute, Scotland
6	19/05/2010	Colonsay, The strand, pod 15, Argyll and Bute, Scotland
7	22/06/2010	Morston Strand, North Norfolk, England

For each shellfish sample, 20 mL crude methanolic extracts were prepared from 2.0 g tissues to provide a solvent-to-sample ratio (SSR) of 10:1 (as described in section 2.4.4). These were then analysed by HPLC-MS/MS and extracts were shown to be free of target toxins (OA, DTX1, DTX2, PTX2, AZA1 and YTX). Prior to spiking these extracts and to maintain the SSR of 10:1, 1.0 mL aliquots (*n*=5) were evaporated to 0.93 mL in 15 mL graduated centrifuge tubes held in a water bath (38°C), using a gentle flow of oxygen-free nitrogen. Evaporated extracts were then fortified by the addition of small volumes of spiking solution. Spiking with OA, DTX1, DTX2 and PTX2 provided concentrations equivalent to 80 µg[toxin]/kg in solution. For AZA1, 40 µg/kg and for YTX, 250 µg/kg concentrations were prepared, respectively. The volumes of spiked extracts were then made up to exactly 1.0 mL by the addition of methanol.

Mean peak area responses were determined for each toxin after HPLC-MS/MS analysis. Also, mean peak area responses derived from analyses of five replicate methanol-based toxin standards prepared at the same concentrations were calculated. Ratios of mean peak area responses of each toxin in mussel or oyster matrix matched standards to those in solvent-based standards were calculated to provide levels of matrix effects. Ratios were determined as percentages observed after analysis of the crude shellfish extracts.

To assess the effect of sample extract clean up in isolating and removing possible co-extracted matrix influences, solid phase extraction (SPE) was applied to the toxin-free mussel and oyster extracts following the method described by Gerssen *et al*. (2009). The authors reported that, in combination with the alkaline LC gradient (as refined and applied throughout the in-house validation scheme reported herein), extract clean up using SPE polymeric sorbents was an effective approach to reducing matrix effects. In brief, the SPE method involved 0.03 g/1.0 mL Strata-X™ SPE cartridges (Phenomenex, Manchester, UK) being activated with 1.0 mL methanol and equilibriated with 1.0 mL methanol:water (70:30

volume/volume). One mL toxin-free shellfish extract was diluted with 2.33 mL water. This was added to the SPE cartridge and the cartridge was washed (to waste) with 1.0 mL methanol:water (20:80 v/v). The cartridge was eluted with 1.2 mL 100% methanol:0.3% (v/v) ammonium hydroxide and eluate was collected. As described above, 1.0 mL cleaned up extracts (*n*=5) of each sample were then gently evaporated prior to toxin spiking. To calculate percentage matrix effects, mean peak area responses established for each toxin analysed from cleaned up extracts were compared to mean responses obtained from solvent-based solutions.

To assess matrix influences after extract dilution and HPLC-MS/MS analysis, replicated (*n*=3) extracts from each mussel and oyster sample were diluted 2.0, 1.5 and 1.25 times with 100% methanol to provide SSRs of 20:1, 15:1 and 12.5:1. As described above and to maintain these SSRs, 1.0 mL diluted extracts were evaporated prior to spiking with the same levels of toxins and then made up to 1.0 mL volumes. Diluted and spiked extracts were then analysed together with a solvent-based standard solution containing the same levels of toxin concentrations. Mean peak area responses were calculated for each toxin in diluted matrix matched standards and standard solutions. From these, percentage matrix influences were determined.

Crude spiked extracts, SPE-cleaned and spiked extracts and diluted and spiked extracts were analysed with solvent-based standards containing the same concentrations of toxins. Percentage matrix effects derived from the extracts were calculated from mean peak area responses of each analyte in relation to mean responses obtained from solvent-based solutions. On a selected sample basis, graphical presentations of percentage matrix effects observed after extract clean up and dilution were prepared with those effects seen in crude extracts with SSRs of 10:1. Visual comparisons were attempted to identify reductions in matrix effects after extract clean up and dilution.

2.5.4. Determination of method limits of detection and quantitation, and working linear ranges.

The limit of detection (LOD) and limit of quantitation (LOQ) of the entire method (extraction *plus* HPLC-MS/MS analysis) were established by practical experimentation and for the following toxins - OA, DTX1, DTX2, PTX2, AZA1, YTX, SPX1 and GYM. Due to the limited availability of reference standards of AZA2 and AZA3, and the unavailability of PTX1, *homo* YTX, 45 OH YTX and 45 OH *homo* YTX, method LOD and LOQ determinations for these MLTs were not performed. Respectively, LOD and LOQ values were expressed as the concentration (µg/kg) of the target analyte that produced a chromatographic signal (peak height) response, three and ten times higher than the chromatographic baseline or background noise surrounding that peak [*i.e*., signal-to-noise (s:n) ratio of 3:1 and of 10:1; International Conference on Harmonisation Tripartite Guideline, 2005].

Initially, s:n ratios were determined from the LC-MS/MS analysis of fortified extracts of shellfish homogenates (SSR 10:1) covering the range of target shellfish species (as conducted in section 2.5.2). This established indicative s:n ratios in relation to expected toxin concentrations. From these relationships, estimations were then made of the concentrations of each toxin required to fortify replicated (*n*=5; 2.0 g), toxin-free (blank) shellfish homogenates which, after extraction and analysis, would provide s:n ratios approximating to 3:1 (LOD) and 10:1 (LOQ). After shellfish homogenate spiking, extraction and analysis, and where toxin peaks were found with s:n ratios of <3 or >5, and/or <8 or >12, the exercise was repeated and shellfish were spiked with higher or lower

toxin concentrations. Where s:n ratios were between 3 and 5, and 8 and 12, concentrations of toxins were determined and LOD and LOQ concentrations were thus estimated. Method LOD and LOQ were reported as mean ± one standard deviation concentrations (µg/kg) values. Within-batch, relative standard deviations (RSD) provided a level of repeatability of determination at these concentrations.

The working linear range of the MS detector for each of the target toxins was then described from these sensitivity values. Linear ranges provide a measure of performance over which the HPLC-MS/MS method can be applied to the analysis of 'real world', toxin-contaminated shellfish samples. The lower value of this range equated to the method LOQ and the upper value was equivalent to the concentration (µg[toxin]/kg[shellfish tissue]) of highest calibration point on the linear regression plot.

2.5.5. Determination of toxin recovery efficiencies of the extraction method.

The performance of the adopted EU-RL (2006) extraction technique in terms of recovering MLTs was assessed by the analysis of fortified and extracted shellfish tissues. Replicate (*n*=7; 2.0 g) homogenates representing each of the eight shellfish species (section 2.2) were spiked at two levels of concentrations. These were equivalent to:

- an upper level of fortification of:
  - 60 µg/kg for OA [equivalent to 38% of the regulatory limit (RL)]
  - 100 µg[toxin]/kg for DTX1, DTX2, PTX2 and AZA1 (63% RL for each toxin)
  - 100 µg[toxin]/kg SPX1 and GYM
  - 250 µg[YTX]/kg (25% RL), and
- a lower level of sample spiking with toxin concentrations similar to method LOQ values. The ranges of expected concentrations after spiking are summarised in Table 4.

Table 4. Range of expected concentrations (µg/kg) of selected MLTs after lower level fortification of shellfish homogenates.

Toxin	Range of 'spiked' concentrations (µg/kg)
OA	45 - 65
DTX1	25 - 55
DTX2	45 - 60
PTX2	5 -20
AZA1	10 - 30
YTX	25 - 100
SPX1	5 - 10
GYM	5 - 25

Fortification was undertaken using calibrated pipettes. Between 0.5 and 2 mL, and up to 4 mL of the 'low,' and 'high' level spiking solutions were added respectively to shellfish homogenates. Homogenates were vortex-mixed for 3 min to integrate the toxin solutions into the shellfish tissues. Extractions took place approximately one hour after mixing. The volume of MeOH solvent used to perform the first extraction step was adjusted to take into account of the total volume of MeOH-based toxin solutions spiked into the test samples. For HPLC-MS/MS batch analysis, extracts and solvent-based MLT calibration solutions were analysed randomly. Recovery was calculated as the relative difference between the observed and expected concentrations. Values of mean percentage recovery *plus* one standard deviation (s.d.) of each toxin/shellfish tissue combination were reported.

Recovery determinations were also performed using a pre-certified reference material [freeze dried mussel tissue (FDMT1)]. This had been produced by IMB (NRC Canada) in collaboration with the Marine Institute (Galway, Ireland) and the Institute for Reference Materials and Measurements (Geel, Belgium). At the time of this work, only indicative values of MLT concentrations were available. The following describes the preparation of this material. Aliquots (0.35 g) of FDMT1 were transferred to 50 mL centrifuge tubes and reconstituted with 1.65 mL water to form 2.0 g wet weight slurry for extraction. Extractions were performed following the EU-RL (2006; section 2.4.4). Over approximately a 12 month period, three batches of FDMT1 consisting of a total of 42 FDMT1 aliquots were extracted and the MLT concentrations determined from solvent-based calibrants. Mean quantities of free OA, DTX1 and DTX2, PTX2, AZA1, YTX and SPX1 were reported and compared to indicative values provided by IMB, NRCC.

2.5.6. Estimation of extraction *plus* analytical method precision; repeatability and within-laboratory reproducibility.

Method repeatability

Undertaken by a single laboratory analyst, repeatability of the entire method involved the fortification of representative shellfish homogenates (section 2.2) with a suite of MLTs followed by extraction and HPLC-MS/MS analysis. Homogenates (n=7; 2.0 g) were spiked to provide the following tissue concentrations:

- 60 µg[OA]/kg (38% of the RL of 160 µg[OA]/kg)
- 100 µg/kg of each of DTX1, DTX2, PTX2, AZA1 (63% RL 160 µg[per toxin]/kg), and
- 250 µg[YTX]/kg (25% RL of 1 mg[YTX]/kg).
- For the non-regulated toxins, spiking concentrations of 100 µg/kg of SPX1 and GYM were used.

Before extractions were performed, a period of one hour was applied following integration of spiking solutions with tissue homogenates via vortex mixing. As described for the recovery studies, the volume of MeOH used for the initial extraction was adjusted to take into account the volume of MeOH-based MLT spiking solution added to the homogenate. For HPLC-MS/MS analyses, shellfish extracts and MeOH-based calibration solutions (containing OA, DTX1, DTX2, PTX2, AZA1, YTX, SPX1 and GYM) were analysed randomly. Method repeatability was expressed as the variation or percentage relative standard deviation (RSD_r) of the mean toxin concentration in relation to the shellfish matrix.

Within-laboratory method reproducibility

Over an eight week period, the within-laboratory method reproducibility (RSD_R) was assessed. This involved three laboratory analysts performing shellfish homogenate spiking, extraction and HPLC-MS/MS analysis on different days. Each operator extracted and analysed a batch of fortified homogenates (n=7) representing each of the eight shellfish species. Thus, for each representative species Σn=21 extracts were prepared from spiked homogenates. Spiking concentrations were similar to those used for method repeatability determinations. Before sample extraction, one hour was applied post-spiking and toxin-homogenate integration. Solvent-based calibration standard solutions and shellfish extracts were analysed randomly.

2.5.7. Assessment of method ruggedness.

Ruggedness of the LC-MS/MS method was determined experimentally and statistically with the deliberate introduction of parameter changes into the method and subsequent assessment of the effects of these changes. A Plackett-Burman design was used to determine the effects of seven key method parameters (Table 5) comparing the single-batch variability of these deliberate variations against the single-batch method repeatability data. The experimental parameters chosen for study were those thought to most likely to affect the performance of the method, including key aspects of both the extraction and instrumental components of the method (Table 6). Homogenates of each shellfish species were spiked at 80 µg/kg for each toxin (YTX at 150 µg/kg) and extracted according to the written method (section 2.4.3). Each sample was subsequently progressed through the ruggedness experiment as described in Table 6.

Table 5. Plackett-Burman experimental design for method ruggedness testing.

Parameter	Experiment number							
	1	2	3	4	5	6	7	8
A or a	A	A	A	A	a	a	a	a
B or b	B	B	b	b	B	B	b	b
C or c	C	c	C	c	C	c	C	C
D or d	D	D	d	d	d	d	D	D
E or e	E	e	E	e	e	E	e	E
F or f	F	f	f	F	F	f	f	F
G or g	G	g	g	G	g	G	G	g
Observed result	s	t	u	v	w	x	y	z

Table 6. Experimental design for ruggedness testing of lipophilic toxins in each shellfish species.

Parameter	Method value	Low value	Parameter code	High value	Parameter code
Extraction volume	9.0 mL	8.8 mL	A	9.2 mL	a
1st extraction vortex mixing time	3.0 min	2 min 50s	B	3 min 10s	b
Centrifugation speed	3500 rpm	3400 rpm	C	3600 rpm	c
2nd extraction mixing speed	11000 rpm	13000 rpm	D	16000 rpm	d
Centrifugation time	8.0 min	7.5 min	E	8.5 min	e
LC flow rate	0.3 mL/min	0.28 mL/min	F	0.32 mL/min	f
pH of mobile phases	pH 11.0	pH 10.8	G	pH 11.2	g

Parameter differences were calculated for each experiment by subtracting the means of the two data sets of results relating to the two variables. For example, the parameter difference for extraction volume (A and a), was calculated by:

$$D_A = [(s+t+u+v)/4] - [(w+x+y+z)/4]$$

where:

s, t, u and v are the experimental results relating to an extraction volume of 8.8 mL, w, x, y and z are the results relating to an extraction volume of 9.2 mL.

The same process was applied to each of the parameters in turn following the experimental approach summarised in Tables 4 and 5. In order to assess the variability of the ruggedness experiment, method repeatability data was utilised from the within-batch precision exercise conducted previously (section 2.5.5). To establish whether such parameter differences were significant and thus result in potential instability of the method, the results were compared against the method precision data using a Student *t*-test.

2.5.8. Evaluation of measurement uncertainty.

For the purposes of evaluating measurement uncertainty (MU) for the determination of MLTs in those shellfish species commonly tested in the UK, method performance characteristics generated from the SLV exercises were used and combined in MU calculations. Where method performance information existed for individual toxins measured in the eight representative shellfish species, those sources of uncertainty in which standardised uncertainties were calculated and then combined, included:

- within laboratory method repeatability (RSD_r)
- within laboratory method reproducibility (RSD_R)
- precision associated with toxin recovery
- variability of toxin signal response as a product of co-extracted matrix influences from seven Common mussel and Pacific oyster tissues taken over time and from different production areas.

Estimations of overall measurement uncertainties were then determined from standardised uncertainty values. Using a coverage factor (k) of two, expanded uncertainties were calculated. This was to provide the dispersion or range of the values within which the value of the quantity being measured is expected to lie (International Organisation for Standardisation, 1995).

2.6. HPLC-MS/MS method transfer and method performance verification.

During mid-2010, Cefas invested in a second LC-MS/MS instrument. This was a Waters Ltd., (Manchester, UK) *Acquity* Ultra-Performance Liquid Chromatograph (UPLC™) coupled to the *Xevo TQ* triple quadrupole mass spectrometer (UPLC-MS/MS). With the application of UPLC technology and small (<2 µm) particle LC columns, significant improvements in analyte resolution, sensitivity and speed of analysis can be achieved for chromatographic separations. Following instrument commissioning, an exercise was conducted whereby several method performance characteristics established during the in-house validation scheme involving the *1100* LC and *Quattro Micro* MS were verified. Method properties such as selectivity, linearity of detection and limits of sensitivity were investigated. By undertaking analytical trials over three and four working week periods, both instruments were trialled on separate occasions. A statistical assessment was made of the instruments' capabilities of analysing the same contaminated shellfish samples on a routine basis by comparing toxin concentrations.

2.6.1. Method transfer and optimisation.

Prior to method verification, toxin analytes were tuned and optimised using the *Xevo TQ* mass spectrometer by applying a similar approach as described in section 2.4.2. Mass spectrometric parameters such as electrospray capillary voltages, source and desolvation temperatures, cone voltages and collision energies were optimised for the production of precursor and two multiple reaction monitoring transition ions per analyte. The conventional HPLC method was then transferred to the *Acquity* UPLC™ instrument. The gradient method was converted to enable compatibility with a <2 µm column (*Acquity* UPLC BEH C_{18}; 2.1 x 50 mm, 1.7 µm; Waters Ltd., UK). Using either individual certified reference standard solutions or contaminated shellfish extracts containing toxins that were unavailable in purified format, the UPLC gradient was further refined and optimised (flow rate 0.6 mL/min; column temperature 30°C) to permit their separation (Table 7).

Table 7. UPLC™ gradient for the separation of regulated and non-regulated MLTs.

Time (min)	A (%)	B (%)
0.0	75	25
1.5	50	50
1.6	50	50
1.7	25	75
4.0	0	100
5.2	0	100
5.3	75	25
6.5	75	25

A: 100% water + 2 mM ammonium hydrogencarbonate (pH 11)
B: 90% acetonitrile:10% water + 2 mM ammonium hydrogencarbonate (pH 11).

2.6.2. Selectivity of the UPLC-MS/MS method.

The approach to assessing the selectivity of the UPLC-MS/MS method was similar to that described in section 2.5.1. Visual examinations were made of each MRM toxin transition chromatogram derived from the analysis of blank extracts representing each shellfish species of interest. The purpose was to identify non-toxin, chromatographic interferences at the expected retention times of the target MLTs.

2.6.3. Linearity of detection.

Linearity of detection of the UPLC-MS/MS instrument was assessed by the analysis of seven levels of calibration standard solutions prepared in methanol solvent and in representative, shellfish extracts [*i.e*., matrix matched standard (MMS) calibration solutions]. Extracts used to prepare MMS were the same as those used for the linearity studies conducted on the *Quattro Micro* mass spectrometer (section 2.5.2.). For the regulated toxins OA, DTX1, DTX2, PTX2, AZA1 as well as the non-regulated compounds SPX1 and GYM, concentrations ranged from 4 to 400% of the 160 µg[toxin]/kg value of the regulated toxins. For YTX, a range of 4 to 400% of the 1 mg[YTX]/kg limit were prepared. For each level of calibration, five repeated LC injections were performed randomly. Correlation coefficient (r^2) values were determined from least-squares, linear regression analyses. The linearity of the method was assessed through visual examination of calibration plots and residuals, together with an assessment of correlation coefficients and lack of fit (see section 2.5.2 for methods of approach).

To evaluate the influence of co-extracted shellfish matrix on toxin signal (peak area) response during LC separation, a comparison of regression equation gradients was made between methanol and shellfish matrix matched standard calibrations. Ratios of gradients derived matrix matched calibrations to those from methanol-based toxin calibrations were calculated. A description of matrix influences, as either enhancement or suppression effects on MLT signal responses, was then made from an assessment of these ratios.

2.6.4. Instrumental limits of quantitation and detection.

The sensitivity of the UPLC-MS/MS method was estimated from the analysis of toxin-spiked extracts of the eight shellfish species of interest. Limits of quantitation (LOQ) were derived from the replicate (n=5) analysis of single extracts. These had been fortified at concentrations equivalent to the method LOQ determined for the *Quattro Micro* mass spectrometer (sections 2.5.5. and 3.2.4.). Using quantitation MRM ions, mean concentrations were calculated for each toxin and signal-to-noise (s:n) ratios were

averaged from peak height responses. By extrapolation and applying s:n criteria of 10:1, LOQ were estimated on a µg/kg basis for each toxin/shellfish combination.

2.6.5. Comparison of performance of two analytical methods.

Throughout 2010, two analytical trials were undertaken using the *Quattro Micro* and the *Xevo TQ* mass spectrometers. Trial 1 took place in April and over three consecutive weeks utilising the former instrument (HPLC-MS/MS). Trial 2 involving the *Xevo TQ* (UPLC-MS/MS) was conducted for four consecutive weeks and between September and October. The objectives of conducting these trials were:

- to develop a robust team of Cefas staff and equip personnel with the necessary skills to enable them to perform, with competence and efficiency, the chain of procedures involved in routine MLT analysis. Procedures ranged from: sample extraction and extract hydrolysis; LC and MS instrument preparation and maintenance; extract analysis; data processing and reporting, and
- particularly from trial 1, to identify any constrictions or limitations in the chain of procedures and to develop refinements and improvements in preparation for trial 2.

Three hundred shellfish samples were analysed in trial 1 and 400 samples were processed during trial 2. For both trials and on a daily basis, 20 samples were prepared as a batch, extracted and analysed. This number is typical of the mid-to-upper range of official control (OC) samples received during the period where lipophilic toxins are normally observed to occur in UK waters. The composition of these included OC shellfish received daily at the Cefas laboratory. Samples were selected from shellfish producing areas from England, Wales and Scotland with most recent or with past histories of contamination by MLTs. Daily to weekly information describing the presence of lipophilic toxin producing dinoflagellates from national phytoplankton monitoring programmes also aided in OC sample selection. During both trials, it was the intention to obtain as many contaminated tissues as possible to develop the training of Cefas analysts particularly in the area of data processing and reporting. Comparisons with concomitant animal assay results associated with target samples were not made.

For each batch of samples and where it was feasible, ten mussels and two Pacific oysters were selected for analysis. Where possible, the remaining three samples were composed of cockle, Native oyster, King or Queen scallop, or Hard or Razor clam species where possible. Five archived (-20°C), naturally contaminated shellfish tissues were included. Predominantly, these materials consisted of Common mussels and several toxin-incurred, Common cockle and Pacific oyster tissues. Prior to each trial, homogenised archived tissues were completely thawed and re-homogenised via Ultra Turrax™ mixing. Aliquots (2.0 g) of each of the materials were transferred to 50 mL centrifuge tubes, anonymously and randomly numbered and stored at -20°C prior to analysis. On the day of analysis, five of these were selected, thawed and prepared together with the OC monitoring samples as a single batch. Some of the archived samples used during trial 1 were included in trial 2 with the objective of comparing MLT concentration data generated from the two instruments.

2.7. Bench marking the performance of the LC-MS/MS method.

During the period of undertaking the in-house validation exercises, Cefas secured opportunities to obtain independent checks of the performance of the HPLC-MS/MS method. By taking part in interlaboratory studies for the determination of MLTs in shellfish

matrices, comparisons of the performance of the method with other laboratories carrying out similar analyses were made possible. Cefas participated in an on-going proficiency programme which is open to all laboratories. Based on our experience in lipophilic toxin analysis and participation in the European Reference Laboratory for Marine Biotoxins working group for such analyses, Cefas was also selected to take part in three collaborative studies. The following provides background to these, including study objectives and a description of the work carried out.

2.7.1. Participation in QUASIMEME scheme for the measurement of lipophilic toxins in shellfish matrices.

Throughout 2009 and 2010 and on a subscription basis, the HPLC-MS/MS method under validation at the Cefas laboratory was applied to the measurement of MLTs in three rounds of performance studies organised and by the programme - *Quality Assurance of Information for Marine Environmental Monitoring in Europe* (QUASIMEME; www.QUASIMEME.org). These were rounds 55 (April to July 2009), 59 (October 2009 to January 2010, and 61 (April to August 2010). The developmental exercises involved the determination of OA, DTXs and AZAs toxins in standard solutions, shellfish extracts and shellfish tissues were undertaken and data were submitted within a set time frame. Total OA and DTX contents were quantified after sample extract hydrolysis (following Mountford *et al*., 2001; section 2.4.4). As DTX1 and DTX2 pre-release reference materials had been made available to Cefas by IMB, NRC Canada, their levels in the test samples were established directly from their respective calibrations. However for AZA2 and AZA3, these were quantified indirectly from AZA1 calibration plots prepared from the commercially available AZA1 certified reference standard.

For each test sample and together with Cefas concentration values, the assigned mean concentrations are presented. The assigned mean were established from consensus values from the participants. The performance of the HPLC-MS/MS method was described in terms of *z*-scores which were calculated from the ratio of the difference between the Cefas value and the assigned concentration, and the target range and are based on the properties of a normal distribution. A score of $|z| \leq 2$ is considered a satisfactory result. A score of between 2 and 3 is regarded as questionable, and $|z| \geq 3$ is considered an unsatisfactory performance. It should be noted *z*-scores take no account of the uncertainties in the assigned value or that of the participant's result.

2.7.2. Participation in the BVL (Germany) method validation collaborative study.

Together with other European laboratories, Cefas participated in the collaborative method validation study coordinated by Federal Office of Consumer Protection and Food Safety (BVL), Germany. The aim of the study was the validation of the quantitative determination of MLTs by LC-MS/MS. The Cefas HPLC-MS/MS method was applied to the measurement of four groups of MLTs (OA/DTXs, PTXs, AZAs and YTXs). Cefas submitted data to the main, collaborative validation study (July to September 2009). In the main study, focus was placed on the extraction and analysis of blind duplicate test samples consisting of cooked mussel tissues and extracts of these, and of spiked crude (raw) extracts of oysters and clams (Table 8). Extraction was performed by a prescribed method involving methanol solvent and a two step Ultra Turrax™ tissue/solvent mixing procedure. Liquid chromatographic separation and mass spectrometric methods remained open to participants to apply their own approaches. Both free and total OA, DTX1 and DTX2 contents of cooked samples were required to be determined. To demonstrate the suitability of the analytical method for uncooked mussel tissues, a second collaborative

study was carried out in April 2010. Due to technical issues with the HPLC-MS/MS instrument at the time, Cefas data was not accepted.

Table 8. Descriptions of the cooked and uncooked samples analysed in the main BVL (Germany) method validation collaborative study (2009).

Sample no.	Sample description
1 (= sample 3)	cooked blue mussels homogenate
2 (Blank sample)	cooked blue mussels homogenate
3 (= sample 1)	cooked blue mussels homogenate
4	cooked blue mussels homogenate
5 (spiked with PTX2)	extract of samples 1 and 3
6 (= sample 8; spiked with OA, PTX2, AZA1 and YTX)	raw oyster-extract, spiked
7 (spiked with OA, PTX2, AZA1 and YTX)	raw clams-extract, spiked
8 (= sample 6; spiked with OA, PTX2, AZA1 and YTX)	raw oyster-extract, spiked

Calibration solutions containing OA, PTX2, AZA1 and YTX were provided together with a single solution containing these toxins in a mussel extract. The purpose of the latter was to examine effects due to matrix influences. Samples were required to be analysed in three separate batches. Each sequence had to fulfil quality control criteria of regression coefficient being ≥0.98 and response drift between two calibration curves ≤25%. Estimations of recoveries were performed from the analysis of spiked extracts. Before statistical analysis of the concentration data, outlier tests (Mandel's statistics, Grubbs test and Cochran test) were applied. Recovery was based on the arithmetic laboratory mean over three sequences of LC-MS/MS analyses.

2.7.3. Participation in the RIKILT (Netherlands) collaborative validation study.

Twelve European laboratories plus one from North America participated in the collaborative validation study organised by the Institute of Food safety (RIKILT), the Netherlands. The study took place throughout the summer and autumn 2010 and its purpose was to determine accuracy, repeatability and between-laboratory reproducibility of the method as described in a written standard operating procedure. Shellfish tissue extraction involved a prescribed three step, vortex mixing method with methanol. Analysis was required to be performed to a prescribed LC-MS/MS method as described by Gerssen *et al.* (2009). The alkaline (pH 11) LC method had been adopted by Cefas and refined for the purpose of undertaking the in-house validation exercises described within this report. Thus, participation provided Cefas with the opportunity to assess the performance of its analytical method in relation to the same method being applied by other international laboratories.

Five mussel, two oyster and one cockle together with blank mussel and oyster tissues were supplied as blind duplicates. The oysters and cockle samples had been blended with between 13 and 50% of contaminated mussel tissue to achieve measurable quantities of toxins in these matrices. Pre-release reference standards including DTX1, DTX2, and AZA2 and AZA3 were provided to enable direct quantititation of these analytes as well as their indirect measurements from respective OA and AZA1 calibrations. Participants were required to prepare their own matrix matched calibration series which included those toxins listed above as well as PTX2 and YTX. The shellfish matrix was a toxin-free, mussel extract sourced and prepared in each participant's laboratory. Each participant thus applied matrix correction during LC-MS/MS analysis from different mussel matrix matched standards (MMS). All samples were individually and randomly numbered per laboratory and the sequence order of analysis was prescribed. As with the BVL

interlaboratory study, the sequence of extract analysis had to fulfil quality control criteria of regression coefficient being ≥0.98 and a response drift of ≤25% between two MMS calibration curves.

2.7.4. Participation in the EU-RL (Spain) interlaboratory validation study.

An interlaboratory study coordinated in 2010 and by the European Reference Laboratory for Marine Biotoxins (EU-RL) involved the 15 participants from 15 European countries. The purpose of the study was to describe accuracy, repeatability and between laboratory reproducibility of the EU-RL harmonised standard operating procedure (SOP) for the determination of lipophilic toxins in bivalve molluscs using LC-MS/MS (EU-RL, 2009). Quantitative determinations OA, DTXs, PTXs, AZAs and YTXs were required from the LC-MS/MS analysis of seven shellfish samples. These included one uncooked mussel tissue, two cooked mussel tissues, a cockle and three clams (wedge shell, razor and striped Venus species). Samples were blind duplicates; some of these were naturally contaminated with MLTs and others had been fortified with toxins.

Shellfish tissue extraction was fixed according to the EU-RL standard operating procedure (see section 2.4.3) and was the same as the method adopted for the purpose of in-house method validation described in this report. Methods of LC-MS/MS analysis remained open for participants to apply their own approaches. Cefas used the analytical method under validation. For the chromatographic separation of target analytes, ten laboratories applied an acidic LC gradient and five used an alkaline gradient. Although a combination of different LC-MS/MS methods were applied throughout the study, quality control and performance criteria were required to be met. These were similar to those described for the BVL and RIKILT interlaboratory exercises (linear regression $r^2 \geq 0.98$; response drift between two sets of calibrations series ≤25%). Additionally, analyte retention time drift of <3%, peak resolution between OA and DTX2 ≥1 and limits of quantitation for OA and AZA1 of ≤40 µg/kg, 50 µg[PTX2]/kg and 60 µg[YTX]/kg were included as criteria.

An IMB NRC Canada, pre-release multi-toxin calibration standard solution containing OA, DTX1, DTX2, AZA1, AZA2, AZA3 and YTX was provided. The same suite of toxins was prepared (by EU-RL) as a single, mussel matrix matched standard (MMS) solution. Toxin determinations were both uncorrected and corrected for matrix effects. Uncorrected concentrations were derived from two sets of calibration standard solutions made in methanol solvent: one prepared with commercially available certified reference (CR) standards - OA, PTX2, AZA1 and YTX; the other prepared from the multi-toxin solution. Concentration data was obtained by direct quantitation and indirectly (*i.e.*, using OA and AZA1 calibration plots to determine DTX1 and DTX2, and AZA2 and AZA3 concentrations, respectively). To correct for matrix influences on toxin quantitation, the analysis of the MMS solution was incorporated into three analytical batches of sample extracts. Additionally, methanol-diluted extracts (1/50 for OA; 1/6 for DTX1; extracted and prepared by participants) of the NRC Canada, CR Mus-b material were applied to OA and DTX toxin measurements. Together with within and between laboratory method precision and HorRat information, Cefas laboratory mean and the assigned mean concentrations (µg/kg) are reported for each sample as MMS corrected, Mus-b corrected (for OA and DTXs only) and uncorrected for matrix effects. Reported concentrations presented by the EU-RL were not corrected for recovery losses and only total OA and DTX concentrations are presented since at the time of reviewing data here, EU-RL had not provided free OA/DTXs to participants in their draft report (EU-RL, 2010).

3. Results and discussion.

3.1. Outcomes of the pre-validation studies.

Prior to the in-house validation of an LC-MS/MS method for the measurement of marine lipophilic toxins (MLTs) in shellfish typically tested in the United Kingdom, preliminary studies were undertaken. These were designed to:

- select and refine a suitable liquid chromatographic method for analyte separation
- optimise the mass spectrometer for the generation of precursor and fragment ions for each toxin
- establish the recovery efficiency of the adopted shellfish extraction procedure, and
- perform an assessment of the chemical hydrolysis procedure.

3.1.1 Selection and refinement of LC separation method.

Based on published information and for the purpose of this single laboratory validation (SLV) scheme reported herein, the alkaline LC gradient reported by Gerssen *et al*. (2009) was selected, trialled and refined. This LC method met the specific objectives of:

- selecting an HPLC method capable of separating twelve EU-regulated MLTs. This suite excluded acylated esterified derivatives of OA and DTXs (*i.e.*, DTX3s) as these are estimated after chemical hydrolysis of crude methanolic shellfish extracts and by measuring total OA and DTX concentrations, and
- acquiring MS data with minimum or no polarity switching (Figure 1) to meet the capability of our MS instrument (*Quattro Micro*, Waters Ltd., Manchester, UK).

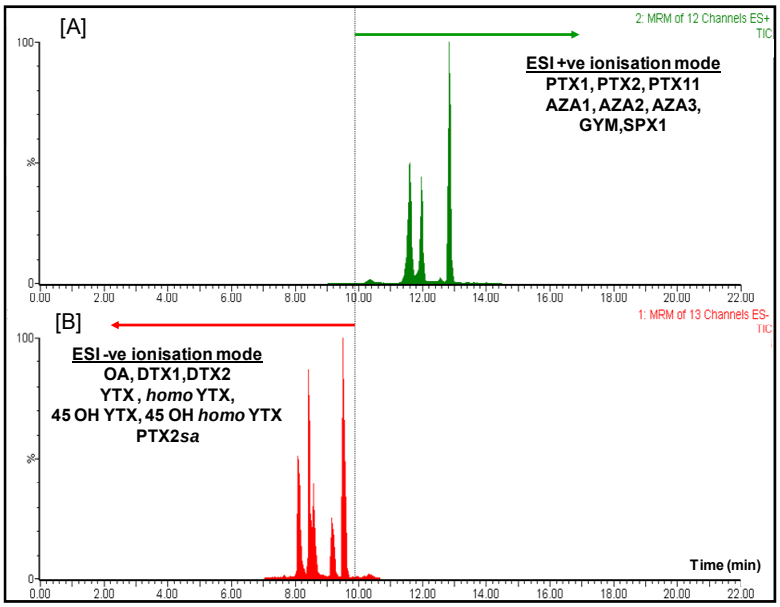


Figure 1. Total ion count (TIC) chromatograms of regulated and non-regulated marine lipophilic toxins amenable to [A] positive electrospray ionisation (ESI) and [B] negative ESI mode using an alkaline (pH11) LC gradient (after Gerssen *et al*., 2009).

An exercise of LC method refinement took place to ensure instrument compatibility. A smaller diameter and smaller pore size *XBridge* C_{18} column (150 x 2.0 mm 3.5 µm; Waters Ltd., Herts., UK) was selected and a lower flow rate [0.3 mL/min opposed to 0.4

mL/min described by Gerssen *et al*., 2009)] was applied. A sample injection volume of 10 µL was used. The lower flow rate was found to be compatible with the *Agilent 1100* LC in terms of maintaining an acceptable back pressure. These changes, in combination with a higher starting percentage (25%:B compared to the published 10%:B) of the acetonitrile mobile phase (Table 9) and a lower column temperature of 30°C (as opposed to 40°C) enabled complete baseline resolution of OA and DTX2 (R_s = 1.6). Although PTX2 and SPX1 can be separated by mass, chromatographic separation was not achieved here and both analytes continued to co-elute. Baseline peak widths of AZA1, 2 and 3 narrowed to ~0.6 min although slight peak fronting was apparent.

Table 9. Optimised HPLC gradient for the separation of target MLTs.

Time (min)	A (%)	B (%)
0.0	75	25
1.0	75	25
11.4	0	100
16.7	0	100
17.0	75	25
22.5	75	25

A: 100% water + 2 mM ammonium hydrogencarbonate (pH 11)
B: 90% acetonitrile:10% water + 2 mM ammonium hydrogencarbonate (pH 11).

As a MS source-compatible, volatile mobile phase buffer, ammonium hydrogencarbonate was chosen in combination with concentrated ammonium hydroxide (for pH adjustment to pH 11) as opposed to using the published 6.7 mM ammonium hydroxide (Gerssen *et al*., 2009). This was due to health and safety reasons as ammonia gas was evident within the immediate vicinity of the LC instrument. A concentration of 2 mM ammonium hydrogencarbonate prepared in both aqueous (A) and solvent (B) mobile phases was found to produce optimal signal responses for OA, PTX2, AZA1 and YTX. Since this salt is fully miscible with acetonitrile and no precipitation is expected at the end of the LC gradient run, the gradient was raised to 90% acetonitrile (equivalent to 100%:B) between 11.4 and 16.7 min; Table 8). It was also observed that as the *XBridge* column aged with continued use and with the use of a lower, end gradient percentage of B as published (*i.e*., 81% acetonitrile), PTX2 was seen to 'bleed' into the chromatogram of the following sample. Increasing the percentage of mobile phase B to 100% eliminated this column effect. All subsequent validation exercises were undertaken using the optimised LC gradient as detailed in Table 9.

3.1.2. Optimisation of mass spectrometric parameters.

Following the refinement of the LC gradient, analytes were separately tuned to establish optimised MS parameters for the generation of a precursor ion of each target toxin, and the production of two multiple reaction monitoring (MRM) transition (fragment) ions for the purposes of toxin quantitation and confirmation.

For OA, DTX1 and DTX2, negative ionisation mode generated the strongest signal responses of the deprotonated $[M-H]^-$ precursor ions. For YTX, the doubly, deprotonated $[M-2H]^{2-}$ ion was selected. Since pure reference standards of *homo* YTX, 45 OH YTX and 45 OH *homo* YTX were unavailable at the time of analyte tuning, the optimised parameters established for YTX were initially adopted for these three analogues along with their precursor $[M-2H]^{2-}$ ions. The MS parameters for these YTX derivatives were further refined using YTX-contaminated extracts from sample extracts listed in Table 2 (section 2.1). The metabolite of PTX2, PTX2 *seco acid* (PTX2*sa*) was also optimised in negative

ionisation mode since its retention time was approx 8 min and eluted within the cluster of OA, DTXs and YTXs.

In positive ionisation mode and for AZA1, AZA2, AZA3, SPX1 and GYM, and for PTX1, 2 and 11 respectively, maximum signal abundances of the protonated $[M+H]^+$ ion and the ammonium adduct $[M+NH_4]^+$ ion were achieved. Cone voltages and collision energies for precursor and transition ions established during analyte tuning and optimisation are detailed in Appendix 2. With the exception of *homo* YTX, 45 OH YTX and 45 OH *homo* YTX, mass spectra of each toxin are present in Appendices 3 to12. Multiple reaction monitoring (MRM) chromatograms are also shown (Appendices 13 to 17) where, with the exception of PTX2sa, chromatograms of two MRM transition ions per toxin are provided. The within-batch performance of the analytical method was assessed and characteristics are presented in Table 10.

Table 10. Percentage relative ion intensities (of base MRM ion peak; highlighted), within-batch variations (%RSD) of peak area responses and relative retention times (RRT; relative to OA) after *n*=30 LC injections of toxin-fortified mussel extract using the alkaline LC gradient.

Toxin	MRM transition	Relative ion intensity (% of base peak)	N° of data points per peak	Peak area variation (%RSD)	RRT variation (%RSD)	Peak width (min)
OA	113.1	44	10	5.2	nd	0.17
	255.3		10	7.0	nd	
DTX1	113.1	57	10	7.7	0.40	0.20
	255.3		10	9.8	0.38	
DTX2 (OA isomer)	113.1	43	10	5.8	0.21	0.25
	255.3		10	5.4	0.23	
PTX1*	213.3	65	8	6.7	0.22	0.15
	821.5		8	6.9	0.22	
PTX11 (PTX1 isomer)	213.3	60	13	2.9	0.21	0.50
	821.5		13	2.8	0.21	
PTX2	213.2	67	12	4.1	0.23	0.25
	823.4		12	4.4	0.23	
AZA1	362.3	84	16	5.9	0.28	0.55
	654.3		16	6.5	0.30	
AZA2	362.5	64	16	7.2	0.31	0.60
	654.5		16	6.6	0.31	
AZA3	362.5	93	12	7.7	0.40	0.55
	658.5		12	7.9	0.38	
YTX	396.2	55	9	10.7	0.38	0.30
	467.2		9	11.9	0.41	
homo YTX	403.4	55	9	12.4	0.38	0.20
	474.4		9	10.6	0.38	
45 OH YTX	396.2	76	9	20.2	0.37	0.20
	467.2		9	14.8	0.35	
45 OH *homo* YTX	403.4	39	8	8.4	0.30	0.20
	474.4		8	6.5	0.30	
SPX1	162.0	71	10	4.6	0.21	0.25
	392.4		10	5.0	0.21	
GYM	444.3	45	9	7.8	0.25	0.25
	177.1		9	3.8	0.20	

* For PTX1, *n* = 7 LC injections were performed. nd = not determined.

The following characteristics were observed:

- peak area signal responses for the two MRM transitions per toxin were established to determine ion ratios. With the exception of OA and its isomer DTX2, and relative to the primary MRM transition peak areas, ion ratios were >50%

- reported as percentage relative standard deviations (RSDs), within-batch variations of relative retention times (to OA; RRTs) of each MLT were 0.2–0.4%. All RRTs complied with the criteria of the RRT margin of ±2.5% as set out in the Commission Decision 2002/657 (Anon. 2002)
- baseline peak widths ranged from 0.15 min to 0.6 min (for AZA2), and
- with the exception of YTXs, peak area RSDs were <10%; for YTXs, RSDs were <20% over the 12 h analytical period.

3.1.3. Toxin recovery efficiencies of the adopted shellfish extraction procedure.

With reference to section 2.4.3., the objective of this pre-validation exercise was to gain information on the performance of the adopted two-step EU-RL (2006) method of toxin extraction from shellfish tissues. To quantitatively recover maximum concentrations of targeted LTs, the method was compared to single, triple and quadruple extraction steps using naturally contaminated mussel tissues.

Extractions involving two or more steps appeared to recover maximum quantities of toxins and only slight differences were found between recovered quantities when double, triple or quadruple extraction steps were applied (Table 11). For DTX1 and AZA1 toxins, mean concentrations were seen to slightly decrease as the more extraction steps were applied. On the basis of the labour, operator time and resources required to perform more than two extraction steps, it was concluded that the double extraction procedure as described by the EU-RL standard operating procedure was acceptable and efficient to quantitatively isolate maximum levels of toxins. This method was then applied to the specific exercises conducted under the SLV scheme.

Table 11. Mean toxin concentrations (µg/kg) [± one standard deviation (s.d.)] recovered from naturally contaminated Common mussel tissues after applying single and multiple extraction steps.

Toxin	Nominal toxin concentration (µg/kg)	Mean ± 1 s.d. toxin conc. (µg/kg; *n*=3) recovered from multiple extraction steps			
		1 step	2 step	3 step	4 step
Free OA	1000	864 ± 11	1000 ± 124	993 ± 79	1048 ± 52
	40	21 ± 5	41 ± 6	41 ± 7	46 ± 7
Free DTX1	600	509 ± 35	603 ± 2	641 ± 61	628 ± 18
	60	49 ± 6	63 ± 2	58 ± 2	55 ± 4
Free DTX2	80	82 ± 9	84 ± 9	90 ± 11	89 ± 0.2
PTX2	10	8 ± 1	8 ± 2	9 ± 0.9	7 ± 0.5
AZA1	4000	3806 ± 186	3962 ± 115	3845 ± 29	3784 ± 14
	5	5 ± 0.7	6 ± 1.2	6 ± 0.3	6 ±1.4
AZA2	700	699 ± 28	738 ± 13	731 ± 15	698 ± 11
AZA3	160	151 ± 13	159 ± 6	159 ± 4	155 ± 4
YTX	110	81 ± 15	103 ± 13	111 ± 17	113 ± 11
	30	31 ± 0.5	34 ± 2	30 ± 1	32 ± 2
45 OH YTX	100	87 ± 12	106 ± 4	101 ± 6	111 ± 9
	30	29 ± 6	28 ± 1	28 ± 2	30 ± 4
SPX1	1	0.93 ± 0.03	1.4 ± 0.07	0.88 ± 0.07	0.73 ± 0.05

3.1.4. Performance assessment of the chemical hydrolysis procedure.

The aim of this pre-validation exercise was to gain information on the performance of the hydrolysis method as described by Mountford *et al*. (2001). Hydrolysis reaction times were investigated in relation to the conversion and recovery of total OA/DTX concentrations (section 2.4.4).

Within 10 minutes (min) after crude extract exposure to hot, alkaline conditions, it was apparent that the conversion of OA (Figure 2), DTX1 and DTX2 acylated esters (Figure 3) to parent toxins was rapid and similar to total toxin quantities produced at 40 min. Those extracts that were not exposed to heating and neutralised at T_0 min contained toxin concentrations equivalent to the free OA/DTX quantities. There was no significant difference in measured total OA or DTX quantities when extracts were hydrolysed for periods of 40 min or longer. Between 40 and 65 min, the variability (relative standard deviation; RSD) of total OA content was between 3 and 9% (average 5.3%) for concentrations approximating to 640 µg/kg. Higher RSDs for total DTX1 (average 13.5%; range 10-17%) and total DTX2 (9.8%; 7-14%) were observed for concentrations approximating to 50 and 60 µg/kg, respectively.

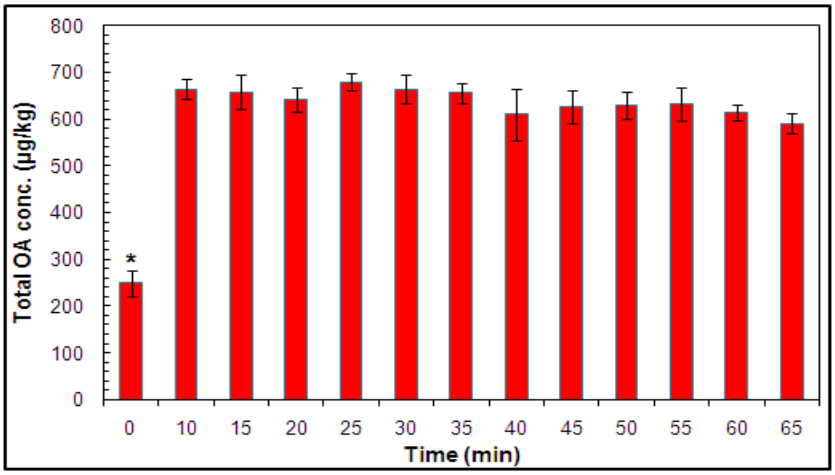


Figure 2. Total OA concentrations (µg/kg) determined in replicated hydrolysed mussel tissue extracts after different chemical reaction times [* indicates significance of difference when compared to 40 minutes reaction time ($p<0.05$, 2-tailed Student *t*-test].

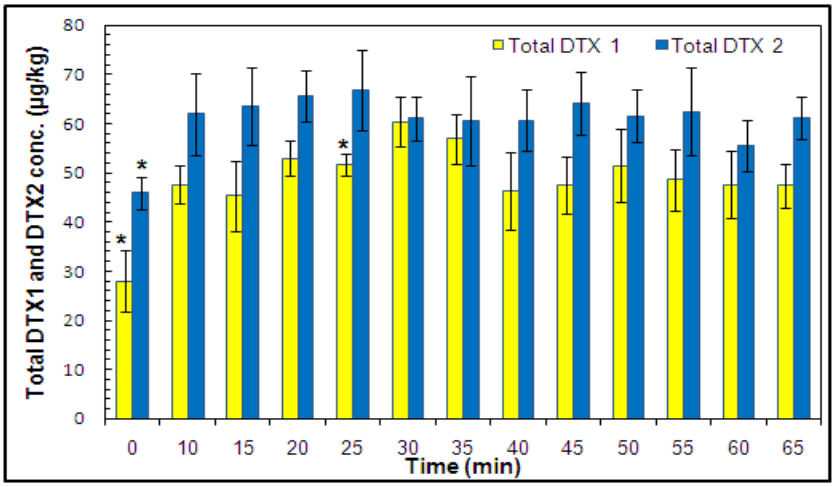


Figure 3. Total DTX1 And DTX2 concentrations (µg/kg) determined in replicated hydrolysed mussel tissue extracts after different chemical reaction times [* indicates significance of difference when compared to 40 minutes reaction time $p<0.05$, 2-tailed Student *t*-test].

Poorer precision associated with toxin determinations is to be expected considering the levels of total DTX1 and DTX2 that were measured. Chemical hydrolysis is an extra procedure in sample extract preparation and may be prone to pipetting (volumetric) errors

associated with transferring crude extracts to vials or with adding sodium hydroxide and hydrochloric acid. Evaporative losses of extracts during heating would also contribute to the precision associated with quantitative results. Combined or alone, these have the potential to incur additional variabilities and which may become apparent when lower µg/kg quantities are to be measured.

It would appear some flexibly can be applied to the length of time (*i.e*., >40 min) that extracts are subjected to hydrolysis. This overcomes the cumbersome practicalities when dealing with a large number of shellfish extracts requiring hydrolysis. It also allows a time margin (*e.g*., within 5 min) after 40 min to remove extracts from heating and to neutralise the chemical reaction following cooling. Importantly, close attention to controlling extract evaporation by the frequent checking of the security of the vial caps is necessary. This can be extended to weighing vial contents after sodium hydroxide has been added and hydrolysis has taken place and after cooling, and prior to neutralization. Correcting for evaporative losses can then be made by the addition of methanol.

3.2. Description of method performance characteristics.

3.2.1 Selectivity of the analytical method.

Selectivity of the established MS/MS detection method was verified by qualitatively comparing chromatograms of toxin-absent (*i.e*., blank) shellfish extracts with chromatograms of toxins prepared in solvent and spiked into a blank extract of Common mussel tissue. The analytical method demonstrated acceptable selectivity. No visible interfering peaks from unknown endogenous and co-extracted substances were observed in shellfish extracts at, or close to the expected retention times, and for the two MRM transition ions chosen for the each of the regulated toxins (OA, DTX1, DTX2, PTX1, PTX2, AZA1, AZA2, AZA3, YTX, *homo* YTX, 45 OH YTX and 45 OH *homo* YTX) as well as the two, non-regulated cyclic imines, SPX1 or GYM. Representative chromatograms of blank shellfish (Common mussel, Common cockle, Pacific oyster, King scallop and Razor clam) extracts and mussel extracts spiked with key toxins including OA, PTX2, AZA1 and YTX reference standards are displayed in Appendices 18 to 21. MRM chromatograms of toxin-free extracts of all of the eight representative shellfish species showed only background signals with low intensities.

3.2.2. Linearity of response from the *Quattro Micro* mass spectrometer.

Regressions constructed for the assessment of linearity of response on the *Quattro Micro* instrument indicated a good degree of linearity for the majority of toxins in each of the matrices examined. Correlation coefficients (Appendix 22) for OA, DTX1/2, PTX2/11, AZA1-3, SPX and GYM all exhibited good evidence of linearity over the entire concentration range as demonstrated with a visual assessment of calibration plots. Examples of selected toxin/shellfish matrix regressions are presented in Appendix 23 and have been prepared from plotting the mean peak area response per level of calibration.

F-test results from the assessment of lack-of-fit in this group of toxins are summarised in Table 12 and show that for the majority of calibrations, there is no statistical indication of non-linearity. Examination of the residuals of the regressions showing potential lack-of-fit furthermore indicated that in all cases the *f*-test failed due to a combination of the effects of a slight bias in a single calibration point (most likely due to small spiking inaccuracies) and lower than average variances of the replicate analyses. A good example of this is the regression for AZA2 in the cockle matrix. Visual examination

of the calibration and the residuals shows good evidence of linearity, but a failed lack-of-fit test results from the very low variability of the replicates and a slight negative residual in the 34.3 ng/mL calibration point (Figure 4). In such instances, use of non-linear (quadratic or polynomial) regressions was not found to result in any improved fit to the regression models. As such, for all these toxins, the linear regression model was demonstrated as being the most suitable.

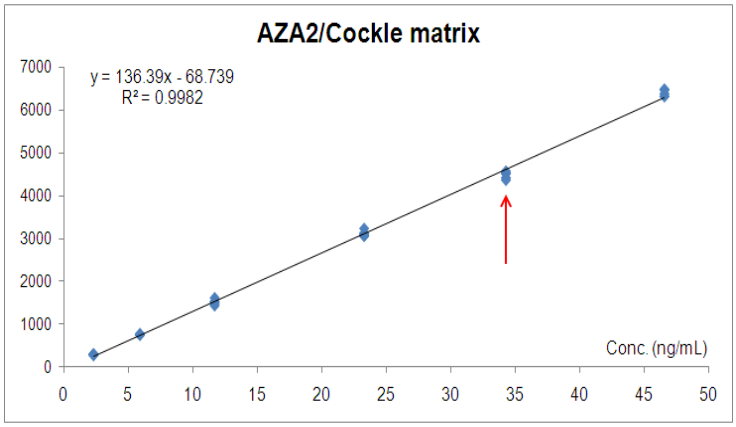


Figure 4. Regression plot of AZA2 in cockle matrix matched standards (arrow indicates slight negative residual at 34.3 ng/mL).

Table 12. Summary of F-test results from assessment of lack of fit on toxin calibrations generated on the *Quattro Micro* instrument.

Matrix	Toxin					
	OA	DTX1	DTX2	PTX2	PTX11	AZA1
Methanol solvent	1.677	0.389	0.703	0.159	0.370	0.894
Mussel	1.047	0.996	2.245	1.557	0.580	1.305
Cockle	0.749	1.678	0.895	0.611	1.186	0.554
Pacific oyster	3.514	0.605	0.335	1.980	2.205	0.916
Native oyster	0.623	1.894	0.720	0.805	0.094	0.649
King scallop	1.108	0.830	1.650	0.694	1.514	1.135
Queen scallop	3.904	0.303	2.142	0.122	0.494	0.868
Hard clam	1.352	0.764	1.493	0.639	0.236	0.313
Razor clam	0.673	2.590	1.192	0.283	1.898	0.639
Matrix	**Toxin**					
	AZA2	**AZA3**	**YTX***	**YTX****	**SPX**	**GYM**
Methanol solvent	2.817	1.057	27.442	3.717	1.128	1.285
Mussel	2.191	0.970	59.235	0.329	2.379	3.854
Cockle	10.009	0.522	73.648	5.141	2.542	3.330
Pacific oyster	0.933	0.037	13.152	1.034	2.232	1.578
Native oyster	0.039	1.045	43.617	0.218	2.762	0.289
King scallop	2.823	2.030	31.081	0.505	0.429	1.530
Queen scallop	0.075	0.159	66.301	0.066	0.450	1.684
Hard clam	0.953	0.115	106.622	1.058	2.157	1.282
Razor clam	0.536	1.386	101.744	0.841	0.810	0.280

F-critical = 2.295 (OA, DTX1/2, PTX2, YTX, AZA1, SPX, GYM).
F-critical = 2.776 (PTX11, AZA2/3).
F-critical = 2.928 (PTX11, Mussels only) and 2.701 (YTX levels 1 to 5 only).
F-values > f-critical are highlighted.
YTX* calibration levels 1 to 6 included. YTX** upper calibration level 6 excluded.

For YTX and in all matrices, strong evidence for non-linearity was found, with a clear curvature in the calibration graphs as illustrated (Figure 5) for YTX prepared in the King scallop matrix extract. Residuals also demonstrate a clear deviation from linearity which in combination with a lack-of-fit shown by failed f-tests (Table 11) unequivocally demonstrates non-linearity.

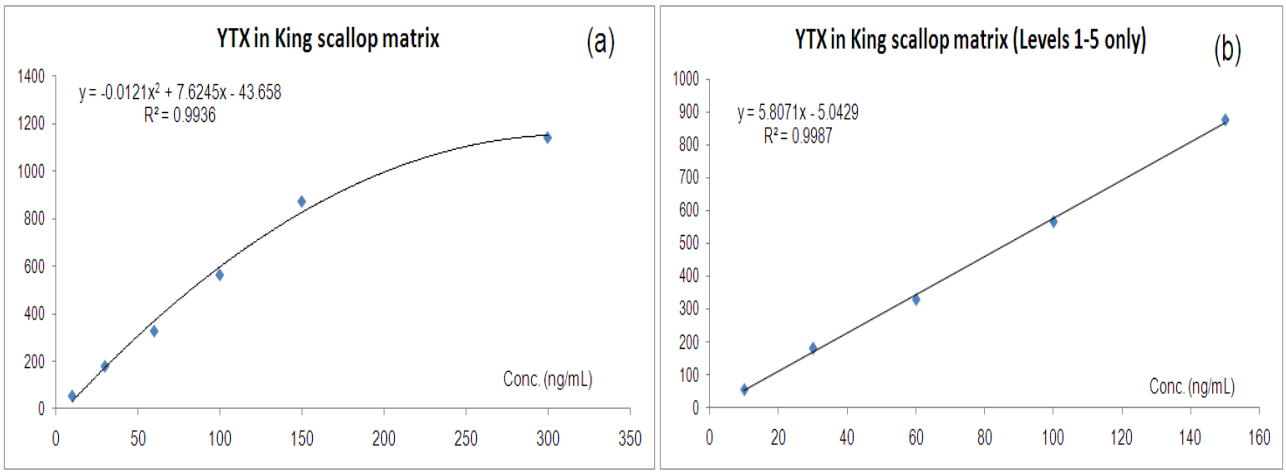


Figure 5. (a) Calibration plot of YTX in King scallop matrix showing non-linearity. (b) linear regression after removal of upper calibration point.

Non-linear regression demonstrated the potential use of quadratic regression for the majority of the YTX calibrations (all quadratic r^2>0.95). However, further examination of the plots revealed the likely source of the non-linearity to result from detector saturation at the highest concentration level (300 ng[YTX]/mL). Removal of this level from the calibrations [Figure 5(b)] subsequently resulted in a linear regression with good correlation coefficients (r^2 > 0.99), no evidence of lack of fit from either the residual plot or the f-test results as shown in Table 11). Consequently, the recommendation from these results was to reduce the upper extremity of linear working range for YTX to 150 ng/mL.

For each of the toxin analytes prepared in methanol solvent and in shellfish extracts representing species commonly monitored in the UK, the linearity of the LC-MS/MS method has been demonstrated. From a combination of correlation coefficient data, visual examination of calibration plots and residuals, together with an assessment of lack-of-fit, evidence for good linear relationships was found. With the exception of YTX on the *Quattro Micro* instrument, the linearity was acceptable in each matrix over the entire concentration range examined for each toxin. Over a concentration range equivalent to 9.4 to 200% of the regulatory limit (RL) of 160 µg[per toxin]/kg, linearity has been demonstrated for OA/DTXs, PTX2/11 and AZA1/2/3 toxins. For YTX, the linear range was equivalent to 10 to 150% of the RL. For the non-regulated toxins SPX and GYM, linear ranges were also acceptable.

Linear plots for MeOH-based calibrations for OA, DTX1 and DTX2, and AZA1, AZA2 and AZA3 toxins are presented in Figures 6a and b. Over the deployed concentration range, OA showed lower signal (peak area) responses in comparison to DTX1 and DTX2 (Figure 6a). Response factors (relative to OA) of DTX1 and DTX2 were similar and approximately 1.7. Similar responses of AZA1 and AZA3 were found although, lower than those exhibited by AZA2 (Figure 6b). The relative response factor of AZA2 to AZA1 (and AZA3) was ~1.4.

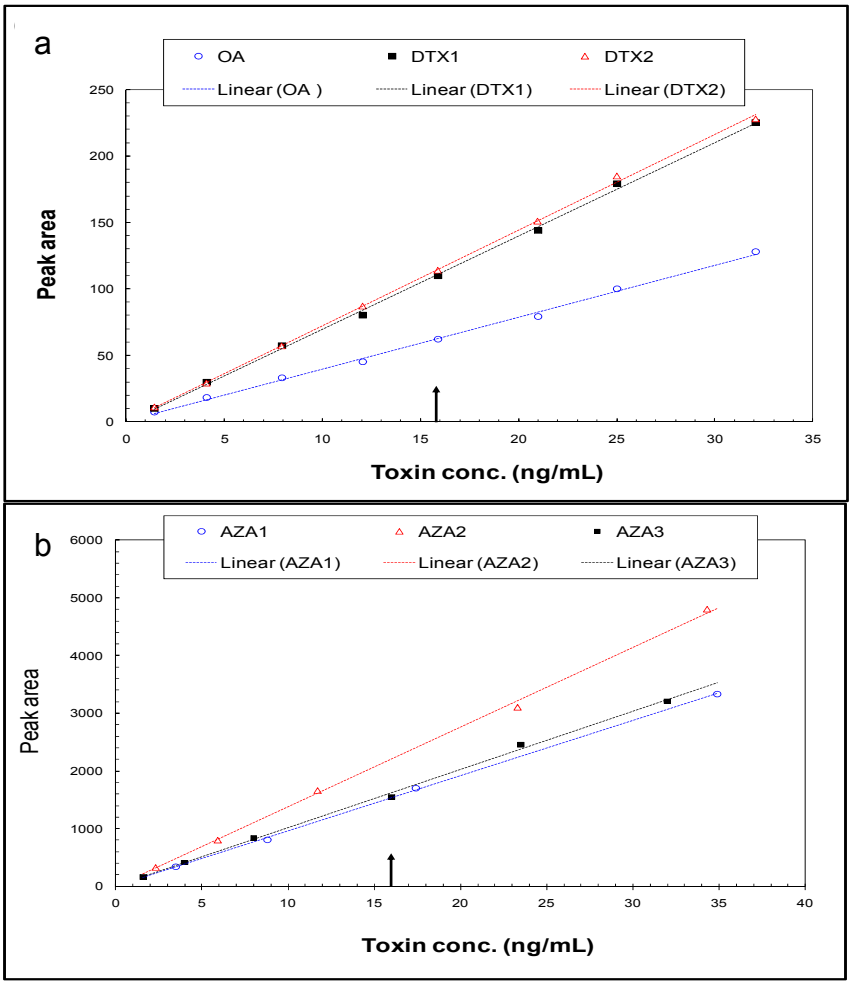


Figure 6. Relationship of regression plots for (a) OA/DTX1/2, and (b) AZA1/2/3 prepared in methanol solvent.
Arrow indicates regulatory limit of 160 µg[toxin]/kg.

3.2.3. Influence of co-extracted shellfish matrix on toxin signal response.

During analyte ionisation, competitive suppression or enhancement effects can occur due to simultaneous elution of analyte-sample extract matrix. This may lead to a bias of quantitation particularly where MLTs are being determined from linear regression plots prepared from solvent-based calibrants. Using gradient data from linear regression equations, an evaluation of these effects was undertaken. Ratios of the calibration gradients derived from matrix matched standard (MMS) calibrations to gradients produced from MeOH-based standard solutions were calculated (Table 12). For YTX, gradients from only linear calibrations were used in calculating ratios. To aid in the evaluation of the degree of matrix influences, the following ranges of ratios were designed:

- negligible effects 0.95 to 1.05 (5% matrix effects)
- weak-to-mild effects 0.90-0.95 and 1.05-1.10 (5-10% matrix effects)
- mild-to-strong effects 0.75-0.90 and 1.10-1.25 (10-25% matrix effects)
- severe effects ≤0.75 and ≥1.25 (>25% matrix effects).

Table 13. Ratios of calibration gradients determined from matrix matched standard solutions to gradients from solvent based standard solutions.

Toxin	Shellfish matrix							
	Common mussel	Common cockle	Pacific oyster	Native oyster	King scallop	Queen scallop	Hard clam	Razor clam
OA	0.87	0.99	0.72	0.84	0.90	0.96	0.86	1.03
DTX1*	0.98	1.14	0.89	0.94	1.10	1.27	1.20	1.35
DTX2*	0.92	1.05	0.89	0.86	0.91	1.11	0.87	1.14
PTX2	0.99	0.77	0.85	0.69	1.06	0.70	0.80	0.67
PTX11**	1.18	1.11	0.73	0.71	0.98	0.85	0.92	0.60
AZA1	1.12	0.84	1.01	0.97	1.01	0.87	0.81	0.94
AZA2*	1.15	0.99	0.85	0.92	0.80	0.81	0.86	0.81
AZA3*	0.90	1.06	0.85	0.89	0.91	0.74	0.84	0.69
YTX	1.30	0.77	1.25	1.26	0.88	1.39	1.44	1.52
SPX1	0.81	0.83	0.71	0.74	0.77	0.75	1.00	0.93
GYM	0.98	0.44	0.90	0.37	0.73	0.49	0.56	0.57

***** Pre-certified reference standards ****** Non-certified reference standard

- With the exception of YTX where a 30% signal enhancement was seen, the mussel extract exerted negligible-to-mild suppression effects on OA/DTXs, PTX2 and AZA3 responses, and mild-to-strong enhancement effects on PTX11 and AZA1/2.
- In the presence of the cockle extract, negligible effects were apparent for OA, DTX2 and AZA2. Where mild-to-strong suppression influences were seen for PTX2, AZA1 and YTX, weak-to-strong enhancement influences were observed for DTX1, PTX11 and AZA3
- For MLTs prepared in the Pacific oyster extract, only AZA1 appeared to be uninfluenced. However, 10 to 25% suppression effects were exerted on DTX1/2, PTX2, and AZA2/3. Where YTX was enhanced by 25%, OA and PTX11 were suppressed by almost 30%
- Strong-to-severe suppression effects were apparent for PTX2/11 when present in the Native oyster extract. As it was seen for Pacific oyster, AZA1 was uninfluenced and YTX was enhanced by 26%. Signal suppression in the range of 6 to 14% was found for OA/DTXs, as well as for AZA2/3
- When present in the King scallop extract, OA/DTXs, PTXs and AZA1 and AZA3, demonstrated negligible or a combination of weak-to-mild signal suppression and enhancement effects. Respectively, YTX and AZA2 were mildly-to-strongly suppressed and by 12 and 20%, respectively
- For toxins prepared in the Queen scallop extract, strong enhancement effects were seen for DTX1 and YTX; strong suppression effects were exerted on PTX2, and AZA3. Where the OA signal was uninfluenced by this extract, DTX2 was mildly enhanced by 11%, and PTX11 and AZA1/2 signals were suppressed by ~15%
- With the exception of DTX1 and YTX where these analytes were found to be enhanced by 20 and 44% respectively, responses of all other regulated MLTs showed weak-to-strong suppressions in the presence of the Hard clam extract
- Signal enhancements were evident for DTX2 (14%), DTX1 (35%) and YTX (52%) in the Razor clam extract. In the range of 31 to 40%, strong suppression effects were apparent for PTX2/11 and AZA3. Only negligible-to-weak effects were found on OA and AZA1
- For the non-regulated lipophilic toxins, GYM appeared to be more susceptible to suppression effects when compared to SPX1.

It was difficult to identify patterns of matrix effects on a single toxin or groups of toxins that were common to a particular shellfish species or groups of species. No robust conclusions could be drawn other than the HPLC-MS/MS method appeared to be prone to a complex array of matrix influences. These ranged from negligible effects, severe signal enhancements such as was observed for YTX and strong suppression influences for PTX2. The levels of signal suppression or enhancement effects found during this exercise are considered to be products of the particular samples used for the preparation of shellfish extracts at the time of this work.

3.2.4. Investigations of shellfish matrix effects on toxin responses during HPLC-MS/MS analysis and approaches to matrix reduction.

The variation of matrix effects on analyte signal responses within crude extracts which were obtained from different samples of the same species (Common mussel and Pacific oyster) and at different sampling times are described (see 2.5.3.). The impact of crude shellfish extract clean up by solid phase extraction (SPE) and extract as practical approaches for reducing the matrix effects exerted on toxins during HPLC-MS/MS analysis were also explored.

Variation of crude mussel matrix effects on toxin responses.

When present in crude mussel extracts, toxin peak area responses differed depending on location of where the sample was acquired and the period of when it was taken. For example and with reference to Figure 7, the mean OA signal response, relative to the mean response found for this toxin prepared in solvent (*i.e.,* 100% as depicted by the dashed line), was mildly influenced in samples 1, 5 and 6. However, a strong (20%) signal suppression was evident in sample 3, and 15 to 20% enhancement was seen in samples 2, 4 and 7. In all sample extracts, DTX1 appeared to be mildly-to-strongly enhanced and in the range of 6-40%. Although weak-to-mild suppression effects were exerted on DTX2 in samples 3 and 6, the response of this analyte was enhanced in all other samples and in the range of 5 to 19%.

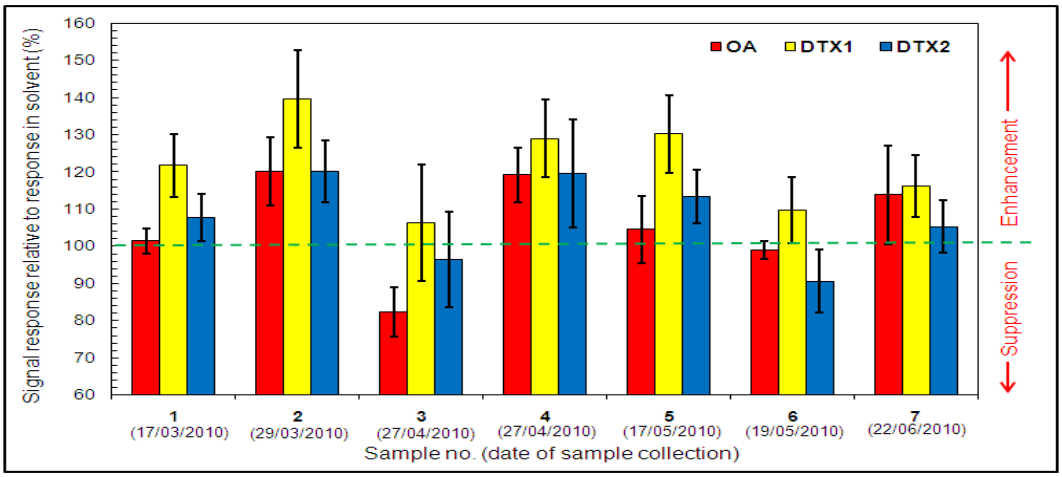


Figure 7. Mean peak area responses of OA, DTX1 and DTX2 in Common mussel extracts obtained from different geographical locations (March to June 2010) relative to mean responses of OA, DTX1 and DTX2 prepared in methanol solvent. Error bars represent ± one standard deviation.

For PTX2, negligible-to-weak matrix influences were seen in samples 4, 5 and 7 (Figure 8). However in sample 1, the toxin was enhanced by 26% and suppressed by 23 and 15% in samples 3 and 6. A strong enhancement of 65% and a suppression of 25% were apparent for AZA1 in samples 1 and 3; all other extracts exerted negligible or weak effects on the response of this toxin. As it was evident for DTX1, YTX suffered mild-to-severe enhancement in all sample extracts and in the range of 7 to 125%.

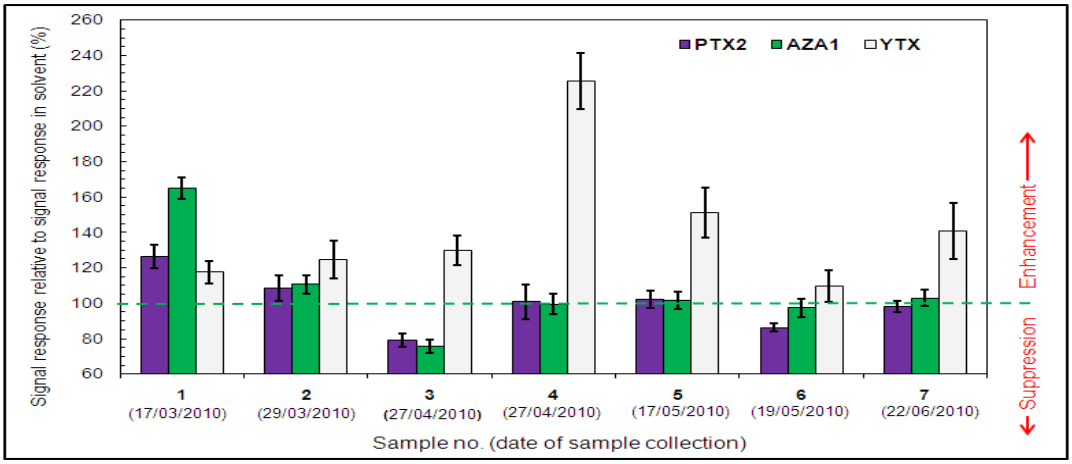


Figure 8. Mean peak area responses of PTX2, AZA1 and YTX in Common mussel extracts obtained from different geographical locations (March to June 2010) and relative to mean responses of PTX2, AZA1 and YTX prepared in methanol solvent (dashed line). Error bars represent ± one standard deviation.

Variation of crude oyster matrix effects on toxin responses.

When present in crude Pacific oyster extracts (Figure 9), the response of OA was affected by similar degrees (~5-15%) of both enhancement and suppression effects. A weak and negligible suppression of DTX1 was seen in samples 2 and 6; all other extracts exerted influences of enhancement in the range of 5-16%. As was demonstrated by OA in sample 6, DTX2 was also suppressed by 16% in the same sample. In other sample extracts, this toxin was found to be mildly enhanced and in the range of 5 to 11%.

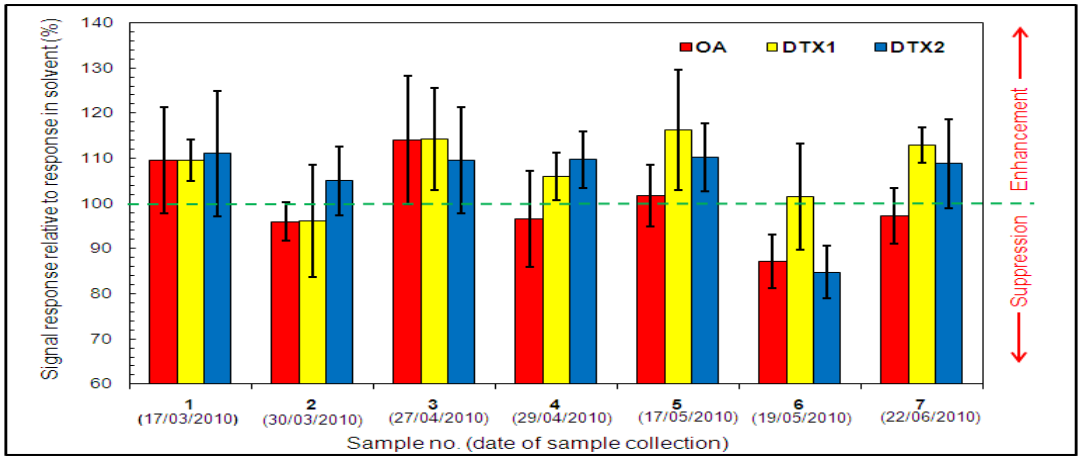


Figure 9. Mean peak area responses of OA, DTX1 and DTX2 in Pacific oyster extracts obtained from different geographical locations (March to June 2010) and relative to mean responses of OA, DTX1 and DTX2 prepared in methanol solvent.
Error bars represent ± one standard deviation.

A range of negligible-to-strong suppression influences on the PTX2 signal were found in most oyster samples with sample 6 showing the strongest effect of 20% (Figure 10). Sample 6 also appeared to induce a suppression effect on AZA1. However, all other extracts exerted a negligible-to-strong (25%) signal enhancement on this analyte. With the exception of sample 6 where a negligible influence was apparent on the response of YTX, this toxin was found to be strongly enhanced in the range of 32 to 76% in the presence of other oyster extracts.

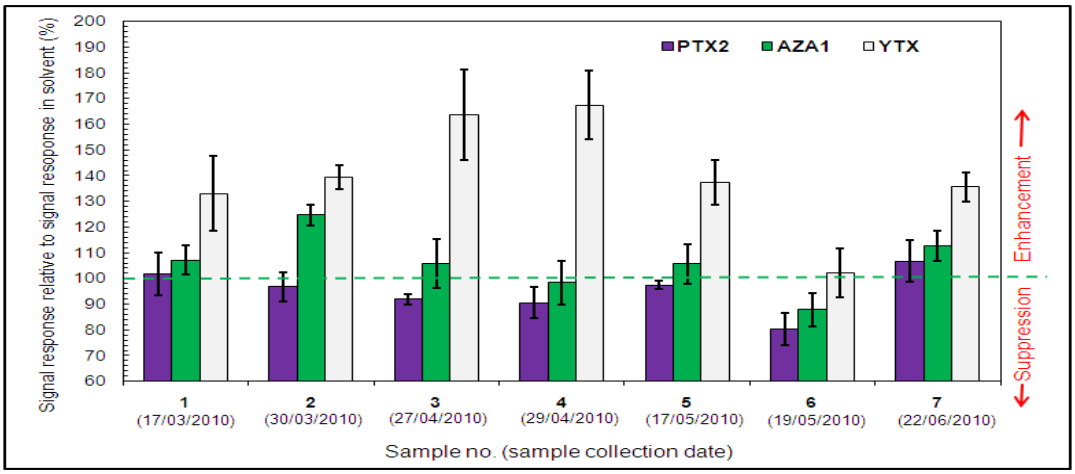


Figure 10. Mean peak area responses of PTX2, AZA1 and YTX in Pacific oyster extracts obtained from different geographical locations (March to June 2010) and relative to mean responses of PTX2, AZA1 and YTX prepared in methanol solvent.
Error bars represent ± one standard deviation.

Removal of matrix interferences from crude mussel extracts

Mussel extract clean up by solid phase extraction (SPE)

With reference to Figure 11 (a-c) and for most mussel samples, extract clean up by SPE appeared to reduce (in the range ~5 to 30%) both enhancement and suppression influences exerted on OA, DTX1 and DTX2. Respectively and for samples 2, 4 and 5, DTX2, OA and DTX1 signal enhancements seen from analyses of crude spiked extracts were eliminated and weak suppression effects of up to 10% followed after clean up. However, extract cleanup of sample 6 had no impact in reducing the suppression effect on DTX2 identified in crude extracts of this sample

Crude extracts of sample 3 resulted in ~15% suppression effects on both PTX2 [Figure 11(d)] and AZA1 [Figure 11(e)], and SPE reduced these to <4%. However for both toxins, the clean up method had only a small impact on removing interfering matrix from crude extracts of sample 1. The response of PTX2 was also suppressed in sample 6 and again by ~15%. After clean up, this level of suppression was found to be elevated to 20%. Where a mean enhancement effect of >125% was seen for YTX in sample 4 [Figure 11(f)], this was reduced to <15% after SPE clean up. This was also reflected in samples 1 and 5 where between 12 and 15% enhancement effects remained after extract clean up.

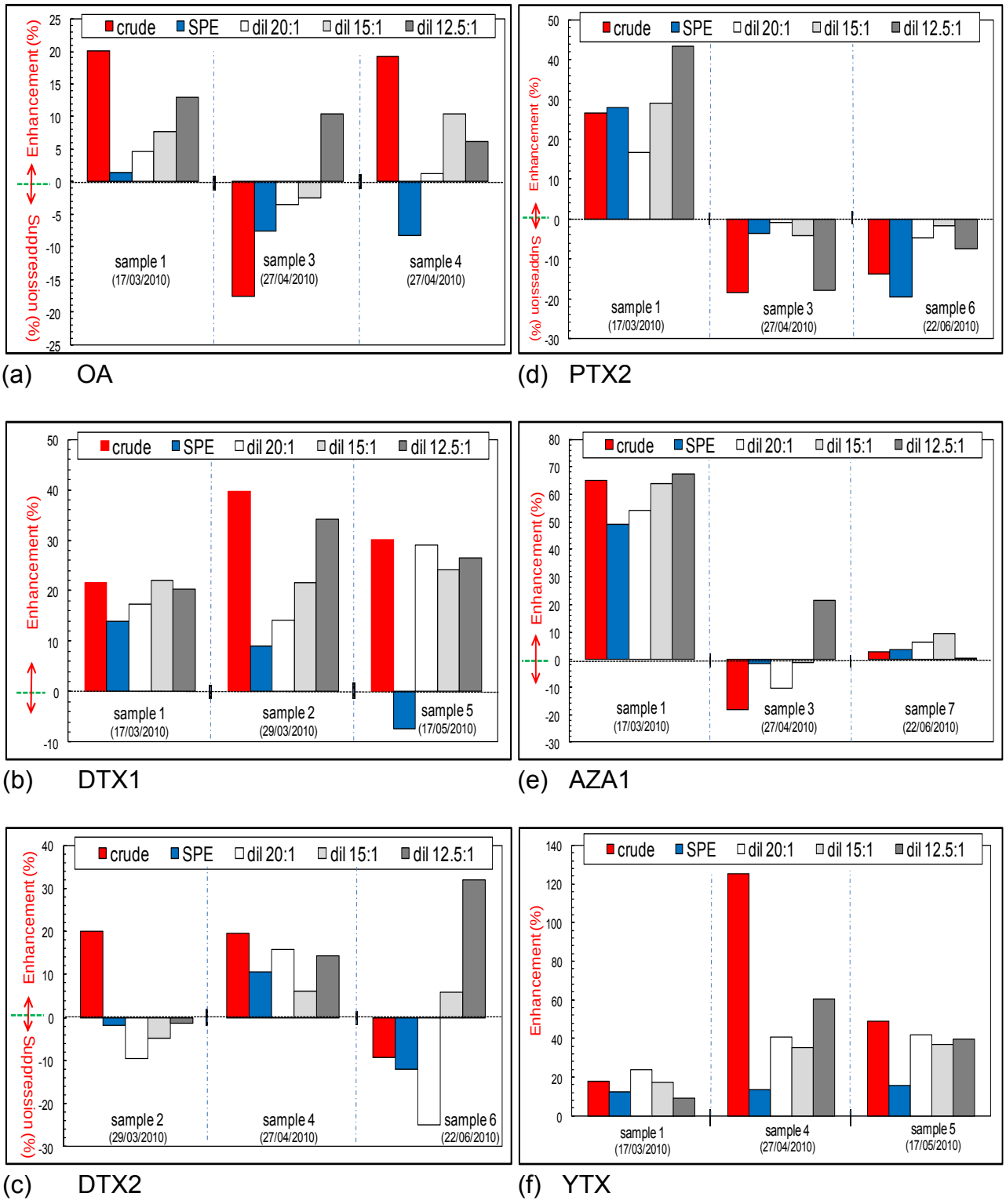


Figure 11. Mean percentage enhancement/suppression effects exerted on lipophilic toxins during LC-MS/MS analysis of spiked crude mussel extracts [crude], cleaned up [SPE] spiked extracts and spiked diluted [dil] extracts with solvent-to-sample ratios of 20:1, 15:1 and 12.5:1.

Mussel extract dilution

Where crude extracts were diluted to provide solvent-to-sample ratios of 20:1, taking this approach reduced either enhancement or suppression effects exerted on OA and in the range of 13 to 18% [Figure 11(a)]. Similarly, this was found for DTX1 in sample 2 and a 35% reduction in enhancement was measured after dilution [Figure 11(b)]. However, lower dilutions resulted in inconsistent patterns of matrix influences for the OA/DTX group. Irrespective of level of dilution, the mean signal responses of DTX1 were not affected in samples 1 and 5. Although all dilutions eliminated a 20% signal enhancement for DTX2 as seen in crude extracts of sample 2, suppression effects of up to 10% resulted after dilution [Figure 11(c)]. Furthermore, the 10% suppression influence on DTX2 from sample 6 was elevated after dilution (SSR 20:1) to 25%. The reverse was apparent after lower dilutions were applied and DTX2 was enhanced by up to 30%.

After extract dilution to provide a SSR of 20:1, matrix influences exerted on PTX2 were found to be reduced. However for AZA1, diluting crude extracts of sample 1 for instance, had little effect in removing a mean signal enhancement of 60% for this toxin. Conversely, the impact of diluting sample 3 crude extracts to provide SSR of 15:1 appeared to eliminate the suppression effects exerted on AZA1. For YTX, dilution of sample 4 crude extracts reduced the severe enhancement exerted on this analyte by ~85%. However, this was not reproduced for samples 1 and 5 where dilution was ineffective in lowering enhancement effects.

Removal of matrix interferences from crude Pacific oyster extracts

Oyster extract clean up by solid phase extraction (SPE)

With reference to Figure 12(a-c), both enhancement and suppression effects influenced OA/DTX signals during LC-MS/MS analyses of crude Pacific oyster extracts. After applying SPE cleanup, enhancement effects were seen to be effectively removed. However, with the exception of DTX1 in cleaned up extracts of sample 5, signal responses of these toxins were found to be suppressed after SPE clean up. For some cleaned up extracts, the resulting levels of suppressions were higher than the levels of enhancements observed from the analysis of crude extracts.

A majority of oyster extracts exerted weak-to-strong suppression effects on PTX2 and in the range of 8-22% [Figure 12(d)]. However, after extract clean up, these suppression influences were larger in proportion compared to levels seen from crude extracts. This was particularly evident for cleaned up samples 3 and 4 where suppression effects on PTX2 increased by factors of ~3 and 2, respectively. The enhancement effect on AZA1 in crude sample 2 was reduced by ~25% by SPE cleanup [Figure 12(e)]. This approach also eliminated the 13% suppression effect seen for AZA1 in sample 6. However, the cleaned extract from sample 7 removed the enhancement effect seen in the crude extract but consequently induced a suppression effect during analysis. This phenomenon was similar to that seen during the analyses of some cleaned up oyster extracts containing OA/DTX toxins. Extract cleanup by SPE was found to be effective in reducing the severe enhancement effects exerted on YTX. Matrix influences were lowered by up to 70% [Figure 12(e)].

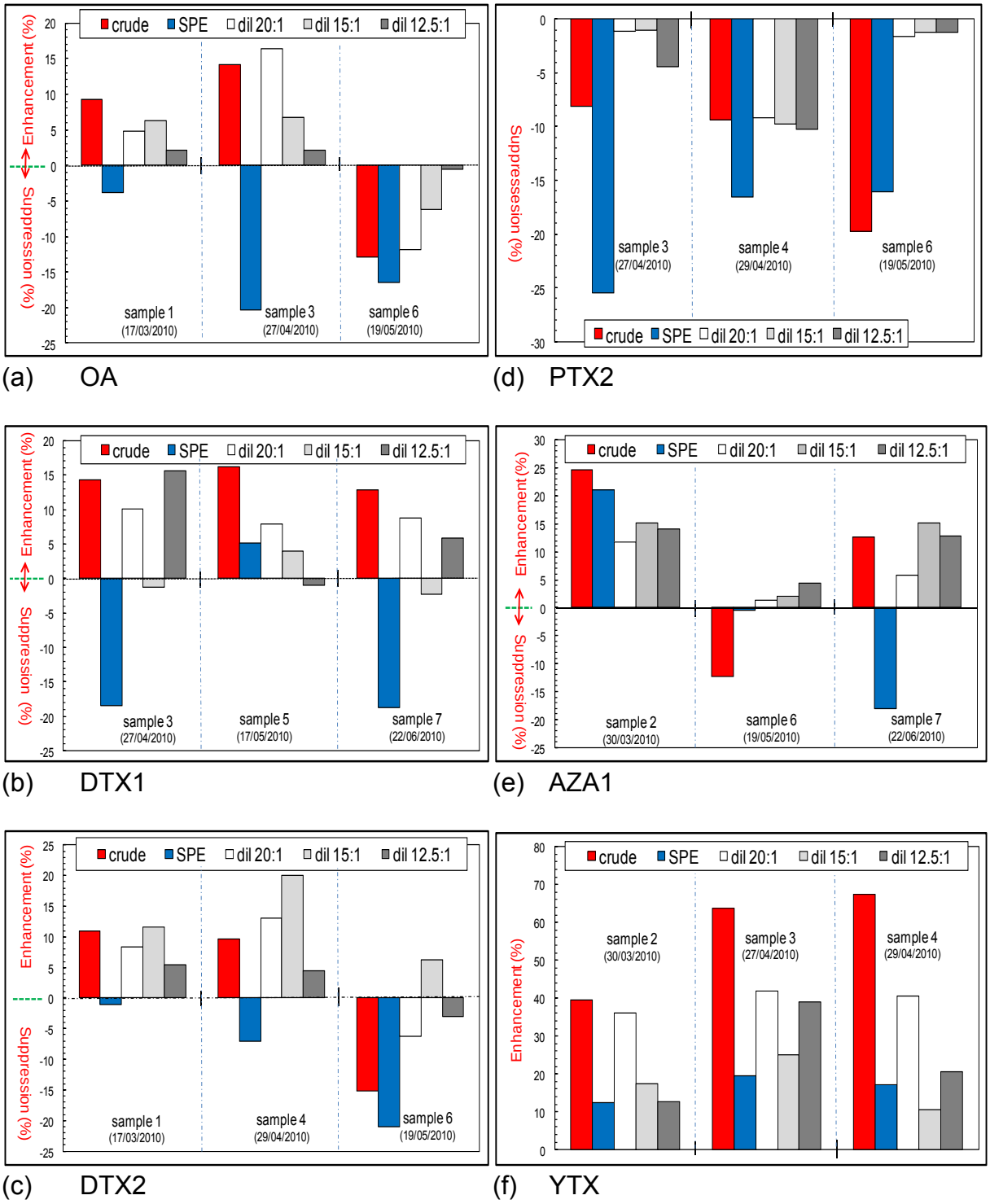


Figure 12. Mean percentage enhancement/suppression effects exerted on lipophilic toxins during LC-MS/MS analysis of spiked crude Pacific oyster extracts [crude], cleaned up [SPE] spiked extracts and spiked diluted [dil] extracts with solvent-to-sample ratios of 20:1, 15:1 and 12.5:1.

Oyster extract dilution

For the OA and DTX toxins, extract dilution resulting in SSRs of 12.5:1 or 15:1 was seen to lower enhancement and suppression effects exerted on these toxins in crude oyster extracts to effects of ~5%. Dilution made little difference to the level of suppression exerted on PTX2 from sample 4. However, from the analysis of crude extracts of samples 3 and 6, the levels of signal suppression exerted on PTX2 were found to be lowered following dilution. Where the mean response of AZA1 was seen to be suppressed in sample 6 by between 10 and 15%, this was seen to be reduced after dilution. Dilution of oyster extracts to provide SSR of 20:1 appeared to be slightly more effective in lowering enhancement/suppression effects on AZA1 compared to other dilution levels. High levels of enhancement influences seen to be exerted on YTX in crude oyster extracts were also reduced by dilution. Where extracts were diluted to provide SSR of 20:1, this approach was generally found to be least effective in lowering the enhancement effects.

The following conclusions were made from the above observations.

- It was apparent that between samples of the same species, there was no similar level of matrix influence for any of the target analytes. Between sample differences in average signal responses for OA, DTX1, DTX2, PTX2 and AZA1 tended to exist and both enhancement and suppression influences analyte responses were observed. However and common to both mussels and oysters, only YTX was found to be enhanced and this ranged from negligible (<5%) to severe (~125%)
- The application of a published extract SPE clean up method was only partially effective in reducing matrix influences on toxins such as OA/DTXs, PTX2 and AZA1. However, reductions were not reproduced for these analytes in all samples. This implies that sample-specific matrix not removed or trapped by SPE may have continued to influence signal responses after clean up and during LC-MS/MS analysis. Particular to oyster extracts, SPE eliminated enhancement influences on OA/DTXs seen in crude extracts. But suppression effects were then subsequently imposed on these toxins. Furthermore, crude extract suppression effects on PTX2 were elevated after SPE clean up
- Evidence found from this study would suggest that SPE clean up, in combination with analysis using the HPLC-MS/MS method, is a possible and practical solution in overcoming co-extracted mussel and oyster matrix influences exerted on YTX. Further work would be required to establish the efficiency of SPE to other shellfish species such as scallops and clams. However, the variable performance of the clean up method makes this option less efficient in reducing matrix influences exerted on other toxins.
- The practice of diluting-out matrix interferences produced a complexity of results regarding reducing effects. There was no common level of dilution that improved matrix reduction effects. There was some evidence that dilution reduced effects on specific toxins, for other toxins, no impact was seen and in some cases, opposite matrix influences were induced after dilution. Due to the variable outcomes seen here, extract dilution would not be a feasible approach to moderating or controlling matrix interferences

3.2.5. Method limits of sensitivity and working linear range.

With reference to section 2.5.4., entire method (*i.e.*, extraction *plus* HPLC-MS/MS analysis) limits of detection (LOD) and limits of quantitation (LOQ) were established by practical experimentation and using the criteria of signal-to-noise ratios of 3:1 (for LOD) and 10:1 (LOQ.). Values are reported in Table 14 as concentrations (µg[toxin]/kg) together with the variation (percentage relative standard deviation; %RSD) associated with these determinations.

Table 14. Method limits of detection (LOD) and limits of quantitation (LOQ) for the determination of selected marine lipophilic toxins.

Toxin	Limits of method sensitivities (µg/kg ± one standard deviation; [RSD %])							
	Common mussel		Common cockle		Pacific oyster		Native oyster	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
OA	7.77 ± 1.69 [21.7]	37.6 ± 7.5 [20.0]	11.5 ± 1.85 [16.0]	49.6 ± 4.5 [9.1]	11.8 ± 0.76 [6.4]	48.5 ± 2.69 [5.6]	11.8 ± 1.41 [11.9]	58.0 ± 5.10 [8.8]
DTX1	14.7 ± 2.0 [13.6]	23.3 ± 3.6 [15.5]	8.86 ± 1.57 [17.7]	46.4 ± 6.2 [13.4]	8.73 ± 1.00 [11.5]	41.9 ± 1.85 [4.4]	11.7 ± 1.60 [13.6]	28.8 ± 3.65 [12.7]
DTX2	10.6 ± 2.0 [18.9]	36.2 ± 5.2 [14.4]	14.0 ± 2.68 [19.1]	43.0 ± 4.6 [10.7]	6.76 ± 1.12 [16.6]	43.5 ± 7.36 [16.9]	14.1 ± 2.63 [18.7]	47.6 ± 1.81 [3.8]
PTX2	2.63 ± 0.14 [5.3]	7.09 ± 0.35 [4.9]	2.26 ± 0.11 [4.9]	7.56 ± 0.54 [7.1]	2.39 ± 0.13 [5.4]	6.13 ± 0.10 [1.6]	3.68 ± 0.19 [5.2]	7.53 ± 0.44 [5.8]
AZA1	2.23 ± 0.16 [7.1]	6.67 ± 0.19 [2.8]	1.43 ± 0.13 [9.1]	4.30 ± 0.28 [6.5]	1.10 ± 0.08 [7.2]	3.78 ± 0.28 [7.4]	3.11 ± 0.08 [2.5]	7.21 ± 0.13 [1.8]
YTX	45.6 ± 1.7 [3.7]	105 ± 16.3 [15.5]	38.3 ± 3.14 [8.2]	69.0± 13.5 [19.6]	5.38 ± 1.08 [20.7]	28.2 ± 3.24 [11.5]	13.8 ± 1.51 [10.9]	48.4 ± 4.02 [8.3]
SPX1	1.13 ± 0.05 [4.4]	2.97 ± 0.15 [5.1]	1.03 ± 0.09 [8.7]	2.54 ± 0.28 [11.0]	0.90 ± 0.06 [6.6]	2.53 ± 0.13 [5.1]	0.51 ± 0.03 [5.9]	2.11 ± 0.08 [3.8]
GYM	1.44 ± 0.35 [24.3]	6.86 ± 0.80 [11.7]	1.57 ± 0.24 [15.2]	7.42 ± 0.85 [11.5]	2.22 ± 0.43 [19.4]	12.0 ± 0.94 [7.8]	5.29 ± 0.52 [9.8]	15.9 ± 2.17 [13.6]
Toxin	**Limits of method sensitivities (µg/kg ± one standard deviation; [RSD %])**							
	King scallop		**Queen scallop**		**Hard clam**		**Razor clam**	
	LOD	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**
OA	16.0 ± 1.98 [12.4]	43.5 ± 2.52 [5.8]	8.69 ±2.05 [23.6]	37.7 ± 5.32 [14.1]	11.1 ± 2.43 [21.9]	57.1 ± 10.3 [18.0]	12.3 ± 3.25 [26.4]	46.7 ± 3.78 [8.1]
DTX1	15.3 ± 3.91 [25.5]	30.5 ± 3.67 [12.0]	9.61 ± 1.82 [18.9]	43.4 ± 4.60 [10.6]	10.8 ± 3.08 [28.5]	36.0 ± 6.23 [17.3]	10.0 ± 2.12 [21.2]	39.0 ± 5.11 [13.1]
DTX2	11.8 ± 2.33 [19.7]	38.7 ± 2.71 [7.0]	10.3 ± 1.93 [18.7]	41.6 ± 6.54 [15.7]	8.18 ± 0.97 [11.9]	40.3 ± 2.58 [6.4]	15.8 ± 2.19 [13.9]	43.8 ± 2.80 [6.4]
PTX2	2.85 ± 0.15 [5.3]	6.91 ± 0.28 [4.1]	3.98 ± 0.21 [5.3]	10.1 ± 0.55 [5.5]	3.41 ± 0.17 [5.0]	5.64 ± 0.38 [6.7]	3.44 ± 0.23 [6.7]	5.95 ± 0.41 [6.9]
AZA1	0.62 ± 0.04 [6.5]	3.17 ± 0.05 [1.6]	3.30 ± 0.23 [7.0]	7.65 ± 0.48 [6.3]	2.59 ± 0.12 [4.6]	7.25 ± 0.43 [5.9]	1.01 ± 0.16 [15.8]	3.23 ± 0.43 [13.3]
YTX	17.1 ± 1.01 [5.9]	51.3 ± 4.00 [7.8]	16.2 ± 0.60 [3.7]	58.1 ± 4.47 [7.7]	13.7 ± 0.80 [5.8]	71.2 ± 7.48 [10.5]	13.9 ± 1.39 [10.0]	56.5 ± 5.53 [9.8]
SPX1	0.95 ± 0.06 [6.3]	2.59 ± 0.17 [6.6]	0.44 ± 0.03 [6.8]	1.19 ± 0.03 [2.5]	0.38 ± 0.03 [7.9]	1.78 ± 0.15 [8.4]	0.34 ± 0.02 [5.9]	1.46 ± 0.08 [5.5]
GYM	3.06 ± 0.46 [15.0]	11.2 ± 2.85 [25.5]	2.85 ± 0.52 [18.2]	10.6 ± 1.95 [18.4]	3.48 ± 0.39 [11.2]	15.2 ± 2.38 [15.7]	3.97 ± 1.10 [27.7]	9.28 ± 1.73 [18.6]

For the OA and DTX group, method LODs ranged from 6.8 µg/kg (DTX2/Pacific oyster tissue) to 16 µg/kg (DTX2/Razor clam). Method LOQs were between 23 (DTX1/Common mussel) and 58 µg/kg (OA/Native oyster). On an individual OA/DTX toxin basis and in relation to the regulatory limit (RL) of 160 µg/kg per individual OA and DTX toxin, LOQs represented 14 to 36% of this value. However, when applying toxicity equivalent factors (TEFs) of 1.0 and 0.6 to convert concentrations of DTX1 and DTX2 to OA equivalents (eq.; EFSA, 2008a), and then summing mean OA/DTX LOQ values as shown in Table 14, ΣOA/DTXs LOQs ranged from 83 µg[OA eq.]/kg (mussel) to 122 µg[OA eq.]/kg (cockle). Respectively, these represent 52 and 76% of the RL of 160 µg[OA eq.]/kg as detailed in Anon. (2004). The extraction *plus* analytical method proved least sensitive for the quantitation of the OA and DTX group in comparison to the lower LOQs achieved for other, key MLTs.

Method LODs for PTX2 for the eight shellfish species were all similar and between 2.3 and 4.0 µg/kg; method LOQs were all ≤10 µg/kg (*i.e.,* ~6% RL). For AZA1, it was observed that King scallop tissue showed the lowest method LOD and LOQ values of 0.62 and 3.2 µg/kg, respectively whereas the Queen scallop tissue demonstrated the highest LOD and LOQ concentrations of 3.3 and 7.7 µg/kg. However, all LOQs for AZA1 were <5% RL of 160 µg[AZA1 eq.]/kg. Comparing LOD and LOQ concentrations to the RL of 1 mg[YTX]kg, LODs for YTX ranged from 5.4 µg/kg (P. oyster) to 46 µg/kg (mussel) and LOQs were <11% RL (range 28 to 105 µg/kg).

The working linear ranges of quantitation *i.e*., the range of the analytical method's ability to achieve peak area responses which are proportional to the concentrations of the target toxin for each individual toxin/shellfish matrix combination are shown in Table 15. On an individual toxin basis and for the OA/DTX group, the linear ranges were from 14 to 200% RL of 160 µg/kg; the lower value being equivalent to limits of quantitation. For PTX2 and AZA1, linear ranges were <10 to 200% of 160 µg[toxin]/kg. With the exception of YTX/mussel tissue combination where a linear range of 0.11 to 1.5 mg/kg were determined, YTX linear ranges were <7.1 to 150% of the 1 mg/kg RL.

Table 15. Linear ranges (µg/kg) of the HPLC-MS/MS method for the quantitation of selected marine lipophilic toxins.

Toxin	Linear range (µg/kg)							
	Common mussel	Common cockle	Pacific oyster	Native oyster	King scallop	Queen scallop	Hard clam	Razor clam
OA	38-320	50-320	49-250	58-320	44-320	38-320	57-320	47-320
DTX1	23-320	46-320	42-320	29-320	31-320	43-320	36-320	39-320
DTX2	36-320	43-320	44-320	48-320	39-320	42-270	40-320	44-320
PTX2	7.1-320	7.6-320	6.1-320	7.5-320	6.9-320	16-320	5.6-320	6.0-320
AZA1	6.7-320	4.3-320	3.8-320	7.2-320	3.2-320	7.7-320	7.3-320	3.2-320
YTX	105-1500	69-1500	28-1500	48-1500	51-1500	58-1500	71-1500	57-1500
SPX1	3.0-320	2.5-320	2.5-320	2.1-320	2.6-320	1.2-320	1.8-320	1.5-320
GYM	6.9-320	7.4-320	12-320	16-320	11-320	11-320	15-320	9.3-320

3.2.6. Assessment of recovery efficiency of the applied extraction method.

Recovery determinations were performed by spiking blank and replicated (*n*=7) representative shellfish homogenates with two levels of concentrations (see 2.5.5.). Higher level fortification corresponded to 60 µg[OA]/kg, 100 µg/kg for DTX1, DTX2, PTX2, AZA1, SPX1 and GYM; and 250 µg[YTX]/kg. Low level sample spiking was performed using analyte concentrations similar to method LOQ values as presented in Table 14. Mean percentage [± one standard deviation (s.d.)] recoveries of target analytes are shown in Table 16.

After extraction of higher level toxin-spiked shellfish, recoveries of all regulated MLTs fell within the range of 74% (YTX from mussel tissue) to 127% (YTX/Razor clam tissue). The majority of the recovery values can be regarded as acceptable and meet the criteria described by the European Commission Decision 2002/657EC *i.e*., -20% to +10% for analyte levels ≥10 µg/kg (Anon. 2002). A mean recovery of 93 % was found for all regulated toxins extracted from all shellfish matrices under investigation. During the SLV scheme described by Gerssen *et al*. (2010) and using a triple (vortexing mixing) extraction technique, mean recoveries of selected MLTs from replicated (*n*=6) and spiked (50% RL) mussel, cockle, Pacific oyster and Razor clam tissues were 93% (PTX2), 97% (AZA1), 94% (YTX) and 97% (SPX1). Average recoveries of the same toxins obtained during the present SLV study and from tissues of the same species were found to be slightly lower,

with the exception of YTX, and were 89% (PTX2), 86% (AZA1), 96% (YTX) and 81% (SPX1). Where tissues were fortified with MLTs at levels close to method LOQ concentrations, the recovery range was between 71% (DTX1/mussel) and 671% (YTX/Razor clam). The exceptionally high recovery of PTX2 and YTX after extracting spiked Hard and Razor clam tissues at levels of 6 and 62 µg/kg, respectively may have been attributed to severe ion enhancement effects exerted on these toxins.

Table 16. Mean percentage recoveries of selected MLTs from 'high' and 'low' spiked shellfish tissues.

Toxin	Mean percentage recovery ± one standard deviation							
	Common mussel		Common cockle		Pacific oyster		Native oyster	
	high	low	high	low	high	low	high	low
OA	82.9 ± 9.9	86.9 ± 11.7	95.3 ± 8.1	103 ± 11.0	86.8 ± 5.1	90.7 ± 8.7	95.1 ± 6.8	118 ± 10.7
DTX1	88.6 ± 5.3	70.8 ± 13.7	91.8 ± 8.9	86.0 ± 7.9	91.5 ± 8.5	92.0 ± 7.2	93.4 ± 6.1	95.0 ± 14.3
DTX2	76.9 ± 7.4	81.1 ± 13.6	92.7 ± 5.9	94.5 ± 4.7	89.2 ± 6.0	98.0 ± 9.7	89.7 ± 5.0	99.0 ± 11.4
PTX2	82.2 ± 4.9	136 ± 15.0	88.1 ± 9.1	95.3 ± 4.7	83.4 ± 3.5	125 ± 11.0	88.1 ± 2.5	137 ± 6.6
AZA1	80.0 ± 3.8	105 ± 11.0	88.4 ± 4.2	91.0 ± 2.0	86.1 ± 3.0	98.6 ± 7.2	80.3 ± 3.1	79.7 ± 6.3
YTX	73.7 ± 3.7	85.0 ± 19.0	89.3 ± 6.6	89.4 ± 6.4	92.5 ± 10.3	80.5 ± 9.0	105 ± 14.1	104 ± 11.2
SPX1	78.8 ± 3.7	70.2 ± 12.5	79.1 ± 5.3	79.7 ± 7.5	76.5 ± 4.2	88.3 ± 8.1	79.9 ± 4.3	70.0 ± 8.0
GYM	82.7 ± 4.2	105 ± 16.0	92.2 ± 2.8	93.8 ± 4.3	86.4 ± 4.4	96.0 ± 9.1	86.9 ± 3.8	117 ± 10.0
Toxin	**Mean percentage recovery ± one standard deviation**							
	King scallop		Queen scallop		Hard clam		Razor clam	
	high	low	high	low	high	low	high	low
OA	97.4 ± 6.8	94.8 ± 3.4	91.4 ± 11.1	83.0 ± 7.0	89.1 ± 4.5	65.3 ± 4.3	100 ± 13.0	97.3 ± 10.7
DTX1	100 ± 5.4	106 ± 6.5	102 ± 9.8	97.0 ± 9.0	110 ± 7.6	82.4 ± 7.9	125 ± 16.0	133 ± 19.0
DTX2	94.9 ± 4.2	98.5 ± 4.8	94.5 ± 7.4	98.9 ± 4.1	95.7 ± 4.5	101 ± 9.2	103 ± 8.7	107 ± 7.5
PTX2	94.0 ± 2.1	95.5 ± 2.1	92.4 ± 3.1	113 ± 8.2	93.4 ± 4.4	199 ± 14	97.3 ± 2.9	122 ± 12.6
AZA1	86.7 ± 1.4	85.9 ± 3.4	90.4 ± 2.1	96.7 ± 2.6	92.2 ± 3.3	49.5 ± 2.7	88.7 ± 2.5	94.8 ± 6.6
YTX	88.9 ± 9.3	91.3 ± 6.4	100 ± 11.1	131 ± 23.0	105 ± 8.3	119 ± 15.0	127 ± 22.0	671 ± 37
SPX1	92.2 ± 4.1	102 ± 9.6	87.9 ± 3.3	115 ± 8.0	87.0 ± 2.6	24.3 ± 4.9	88.7 ± 4.9	56.4 ± 5.1
GYM	96.1 ± 5.1	95.9 ± 5.5	89.5 ± 5.0	99.6 ± 11.0	87.6 ± 4.9	38.5 ± 4.6	95.3 ± 3.7	100 ± 17.0

An evaluation of recovery efficiencies was also undertaken using a pre-certified and pre-released reference material supplied by IMB, (NRC, Canada). This involved the extraction and analysis of rehydrated freeze dried mussel tissue (FDMT1; see 2.5.5). Preliminary concentrations (µg[toxin]/g dry weight; Table 17) were supplied with the material although no level of precision associated with these quantities were provided. Over a 12 month period, batches of FDMT1 were extracted following the adopted procedure (EU-RL, 2006; see 2.4.4).

Table 17. Mean percentage recoveries of selected MLTs from the pre-certified and pre-released freeze dried mussel tissue (FDMT1) reference material.

Toxin	Pre-certified concentration (µg/g dry weight)	Expected equivalent toxin conc. after reconstitution (µg/kg wet weight)	Mean recovery (*n*=42) (%)	RSD (%)
OA	1.2	210	122	9.1
DTX1	1.1	193	82.6	13.7
DTX2	4.1	718	88.6	8.9
PTX2	0.94	165	50.3	20.0
AZA1	3.3	578	111	18.0
AZA2	0.77	135	99.0	15.6
AZA3	0.87	152	71.4	11.1
YTX	2.8	490	88.1	28.8
SPX1	1.0	175	192	20.4

For the EU-regulated toxins, recoveries ranged from 50% (PTX2) to 122% (OA). Recoveries were found to be acceptable although OA, PTX2 and AZA3 did not meet the minimum criteria set out in Decision 2002/657EC. It is interesting to note the poor recovery obtained for PTX2 when compared to satisfactory efficiencies achieved when this compound was spiked to wet shellfish matrices and extracted. One possible explanation may be a product of severe suppression effects due to co-eluting matrix interferences which may be associated with the FDMT1 material. Conversely, and for the non-regulated toxin, SPX1 (13-desmethyl spirolide C), >190% recovery was found. This analyte chromatographically co-elutes with PTX2 and again, competitive matrix interferences associated with the reference material may have enhanced in-source ionisation of SPX1 leading to an overestimation of recovered quantities. Until certified reference concentrations are published for the FDMT1 material, the performance of the adopted extraction method in terms of recovery efficiencies of toxins from this material is to be regarded as indicative.

3.2.7. Estimation of method repeatability and within-laboratory method reproducibility.

Estimation of method repeatability for MLT determinations involved one laboratory analyst performing shellfish tissue spiking, extraction and HPLC-MS/MS analysis (see section 2.5.6.). Fortification was in the range of 60-250 µg[toxin]/kg as well as at concentrations similar to method LOQs. For estimations of within-laboratory method reproducibility, the higher level concentrations were used for spiking shellfish matrices. Over an eight-week period, three laboratory analysts performed the procedures on separate occasion. Values of method repeatability (RSD_r) and the within-laboratory method reproducibility (RSD_R) are provided in Table 18.

Table 18. Method repeatability (RSD_r) and within-laboratory method reproducibility (RSD_R) for the determination of regulated and non-regulated MLTs from spiked shellfish tissues. Data in parenthesis are method repeatability values calculated for MLTs spiked at method LOQ concentrations.

Toxin	Relative standard deviation (RSD; %)							
	Common mussel		Common cockle		Pacific oyster		Native oyster	
	RSD_r	RSD_R	RSD_r	RSD_R	RSD_r	RSD_R	RSD_r	RSD_R
OA	11.9 (13.4)	9.6	8.5 (10.5)	11.1	5.9 (9.6)	9.4	7.2 (9.0)	7.3
DTX1	5.9 (19.4)	5.0	9.7 (9.1)	8.8	9.3 (7.8)	6.9	6.5 (15.1)	4.9
DTX2	9.6 (16.8)	8.9	6.3 (5.0)	6.5	6.7 (9.9)	8.0	5.6 (11.5)	7.3
PTX2	5.9 (10.7)	9.2	10.3 (4.9)	6.4	4.2 (7.3)	4.3	2.8 (4.8)	4.0
AZA1	4.8 (10.0)	4.0	4.7 (2.2)	6.7	3.5 (7.3)	4.9	3.9 (8.0)	4.4
YTX	5.0 (21.9)	15.4	7.4 (7.2)	21.2	11.1 (11.2)	13.7	13.4 (10.8)	12.7
SPX1	4.7 (17.8)	7.7	6.8 (9.4)	5.1	5.5 (9.2)	5.3	5.4 (11.4)	3.7
GYM	5.1 (15.2)	3.1	3.0 (4.6)	5.7	5.0 (9.4)	3.8	4.4 (8.6)	4.2
Toxin	**Relative standard deviation (RSD; %)**							
	King scallop		Queen scallop		Hard clam		Razor clam	
	RSD_r	RSD_R	RSD_r	RSD_R	RSD_r	RSD_R	RSD_r	RSD_R
OA	7.0 (3.6)	8.1	12.1 (7.9)	6.9	5.1 (6.6)	6.5	13.0 (10.9)	7.1
DTX1	5.4 (6.0)	6.7	9.6 (9.4)	4.9	6.9 (9.7)	4.2	12.6 (14.2)	6.2
DTX2	4.5 (4.9)	4.8	7.8 (4.1)	7.0	4.7 (9.1)	4.8	8.5 (7.2)	6.6
PTX2	2.3 (2.3)	3.7	3.4 (7.2)	4.0	4.7 (7.0)	8.8	3.0 (10.2)	7.8
AZA1	1.6 (3.9)	3.3	2.3 (2.7)	4.7	3.6 (5.4)	10.7	2.9 (7.1)	4.3
YTX	9.6 (7.0)	21.1	11.0 (17.5)	11.9	7.9 (12.6)	16.5	17.3 (5.6)	8.6
SPX1	4.4 (9.3)	3.2	3.7 (9.0)	6.8	2.9 (19.9)	8.4	5.6 (8.8)	5.3
GYM	5.3 (5.7)	5.2	5.6 (11.1)	6.2	5.6 (11.1)	4.4	3.9 (17.1)	4.0

For shellfish tissues spiked with MLTs in the range of 60 to 250 µg/kg, RSD_r values were found to be from 1.6 to 17.3% for both regulated and non-regulated toxins. Where

analyte concentrations were determined at the lower extreme range of operation, the variation associated with the determination of MLTs reflecting method LOQs were generally higher for analytes extracted from spiked mussel, Pacific and Native oyster, King scallop and Hard clam tissues. For these shellfish species, it would appear that precision of determination varied with analyte concentration. Repeatability was least affected when both 'high' (60-250 µg/kg) and 'low' (2.5 to 50 µg/kg) toxin levels were quantified from spiked cockle tissue. From the SLV study reported by Gerssen *et al*., (2010), average RSD_r values from the analysis of spiked (at 50% regulatory limits) mussel and Pacific oyster extracts (as opposed to shellfish tissues) were calculated to be 7.2% for OA, 12.0%(PTX2), 5.4%(AZA1), 3.7%(YTX) and 4.3%(SPX1). Overall, RSD_r values found in the current SLV study and presented here were similar in magnitude to those published data. However, both RSD_r and RSD_R calculations obtained for the determination of PTX2 were <50% of those values presented by Gerssen *et al*., (2010).

For within-laboratory method reproducibility, RSD_R data were found to be in the range of 3.1 to 21.2%. As it was seen for estimations of method repeatability, the determination of YTX also demonstrated higher variations. An average RSD_R of 15.1% was calculated for the quantitation of this analyte in all shellfish species. However, considering the range of concentrations of 60 to 250 µg/kg, RSD_R values were in accordance with the reproducibility requirements of the Commission Decision 2002/657 *i.e*., from 23% and up to 32% (Anon. 2002). With the exception of YTX, values presented in Table 18 were similar to those reported by Gerssen *et al*., (2010) although the authors reported their precision values from the LC-MS/MS analysis of spiked mussel and Pacific oyster extracts rather than tissues. Average RSD_R measurements were reported by Gerssen *et al*., (2010) as 10.1% for OA, 17.5%(PTX2), 7.0%(AZA1), 7.2%(YTX) and 6.0%(SPX1). For the extraction, analysis and quantitation of YTX, both method repeatability and within-laboratory reproducibility data determined in this current study were more than twice those published by Gerssen *et al*., (2010).

3.2.8. Ruggedness of the HPLC-MS/MS method.

With reference to section 2.5.7., main effects were calculated as the difference of means for each paired set of parameter levels (*i.e*., parameter differences) and compared to method precision (single batch; n=7) using a two-tailed t-test, and specifically the standard deviations of the within-batch repeatability analysis (RSD_r; Table 18). The *t*-test results for each parameter in each of the eight shellfish matrices are summarised in Appendix 24. The results show that all *t*-test values were lower than *t*-critical, inferring that none of the ruggedness parameters considered had a statistically significant effect on the stability of the method, with the assumption that parameters investigated do not interact. It was noted that variations associated with the ruggedness exercises (Table 19) were lower for OA, DTX and YTX toxins than the values associated with the within-batch repeatability studies. The reverse was apparent for PTX2 and AZA1 where ruggedness RSDs were seen to be slightly elevated above RSD_r values.

It is recognised that the ruggedness study was conducted approximately ten months after the repeatability study. During such time, factors such as laboratory operator skills in sample spiking and extraction may have improved the overall performance of the method. This aside, it is evident from the RSDs obtained from the ruggedness experiments that a low level of variability was apparent following the deliberate variation of the experimental parameters. Overall, the HPLC-MS/MS method was shown to be rugged for all the selected regulated toxins in each of the eight shellfish matrices with respect to the parameters investigated, taking the assumption that the parameters do not interact.

Table 19. Comparison of relative standard deviations (RSDs) calculated from method repeatability and ruggedness exercises.

Toxin	Shellfish species	Ruggedness RSD (%)	Repeatability RSD_r (%)	Toxin	Shellfish species	Ruggedness RSD (%)	Repeatability RSD_r (%)
OA	Common mussel	5.4	11.9	PTX2	Common mussel	4.5	5.9
	Common cockle	5.9	8.5		Common cockle	8.3	10.3
	Pacific oyster	2.9	5.9		Pacific oyster	6.2	4.2
	Native oyster	4.0	7.2		Native oyster	6.0	2.8
	King scallop	2.0	7.0		King scallop	2.4	2.3
	Queen scallop	5.5	12.1		Queen scallop	5.2	3.4
	Hard clam	6.5	5.1		Hard clam	5.2	4.7
	Razor clam	3.4	13.0		Razor clam	3.6	3.0
DTX1	Common mussel	6.2	5.9	AZA1	Common mussel	1.8	4.8
	Common cockle	3.0	9.7		Common cockle	5.1	4.7
	Pacific oyster	2.0	9.3		Pacific oyster	1.7	3.5
	Native oyster	3.9	6.5		Native oyster	3.9	3.9
	King scallop	5.4	5.4		King scallop	4.7	1.6
	Queen scallop	1.8	9.6		Queen scallop	4.7	2.3
	Hard clam	5.7	6.9		Hard clam	4.3	3.6
	Razor clam	5.3	12.6		Razor clam	4.8	2.9
DTX2	Common mussel	3.3	9.6	YTX	Common mussel	5.1	5.0
	Common cockle	3.3	6.3		Common cockle	5.4	7.4
	Pacific oyster	3.1	6.7		Pacific oyster	5.5	11.1
	Native oyster	4.3	5.6		Native oyster	5.4	13.4
	King scallop	1.7	4.5		King scallop	3.7	9.6
	Queen scallop	3.4	7.8		Queen scallop	12.0	11.0
	Hard clam	3.3	4.7		Hard clam	7.3	7.9
	Razor clam	4.9	8.5		Razor clam	6.3	17.3

3.2.9. Estimation of measurement uncertainty.

Uncertainty of method repeatability

With reference to section 2.5.8., measurement uncertainty (MU) evident from the within laboratory method repeatability exercise was evaluated from the statistical distribution of RSD_r data (Anon. 2000), and a normal distribution of values was assumed. Standardised uncertainties were calculated from RSD_r values established after spiking shellfish species with two levels of concentrations (Table 18; section 3.2.7.). Uncertainties represented the variability associated with the combined (extraction *plus* analytical) methods. Using equation 1, the standardised uncertainties at the two levels of spiking concentrations were pooled to provide total standardised method repeatability uncertainties and these are reported in Table 20.

Equation 1:

$$u_c(y) = \sqrt{\frac{(n_a - 1) \times a^2 + (n_b - 1) \times b^2}{(n_a - 1) + (n_b - 1)}}$$

where:

$u_c(y)$ = pooled uncertainty of precision uncertainty components
a,b = RSD_r values of components at two different concentrations
n = number of replicates used in method repeatability studies for each component.

Table 20. Summary of standardised uncertainties associated with the method repeatability for the determination of regulated and non-regulated MLTs in test shellfish species.

Toxin	Common mussel	Common cockle	Pacific oyster	Native oyster	King scallop	Queen scallop	Hard clam	Razor clam
OA	0.127	0.096	0.080	0.081	0.056	0.102	0.059	0.120
DTX1	0.143	0.094	0.086	0.116	0.057	0.095	0.084	0.134
DTX2	0.137	0.057	0.085	0.090	0.047	0.062	0.072	0.079
PTX2	0.086	0.081	0.060	0.039	0.023	0.056	0.060	0.075
AZA1	0.078	0.037	0.057	0.063	0.030	0.025	0.046	0.054
YTX	0.159	0.073	0.112	0.122	0.084	0.146	0.105	0.129
SPX1	0.130	0.082	0.076	0.089	0.073	0.069	0.142	0.074
GYM	0.113	0.039	0.075	0.068	0.055	0.088	0.088	0.124

Uncertainty of within laboratory method reproducibility

Measurement uncertainties associated with within laboratory method reproducibility were estimated from the RSD_R data generated by the repeated extraction and HPLC-MS/MS analysis of spiked shellfish tissues over an intermediate period of time (Table 18; section 3.2.7.). For a selection of toxin analytes, RSD_R values acquired from the repeat extraction and analysis (conducted over a three week period) of naturally contaminated shellfish samples (see Table 31; section 3.3.4) were also incorporated in calculations of standardised uncertainties. Additional method reproducibility data for a number of MLT determinations from a mussel matrix was obtained from the long term, repeat extraction and analysis of a pre-certified FDMT1 reference material (Table 17; section 3.2.6.).

Notably for mussel tissues, a number of RSD_R values were calculated from the measurement of MLTs at different toxin concentrations. In these instances, the total, standardised uncertainties associated with within laboratory method reproducibility were determined by pooling the individual uncertainties as described for MU associated with method repeatability. It should also be noted that the majority of the reproducibility data generated from naturally contaminated tissues related to mussels, with a low number derived from cockles and Pacific oysters. As such, most of the data generated for the other species was obtained only from spiked tissues, which may not accurately reflect the precision inherent in the method for analysis of naturally incurred shellfish samples. The contribution of reproducibility to the measurement uncertainty is summarised in Table 21.

Table 21. Summary of standardised uncertainties associated with within laboratory method reproducibility.

Toxin	Common mussel	Common cockle	Pacific oyster	Native oyster	King scallop	Queen scallop	Hard clam	Razor clam
OA	0.115	0.111	0.094	0.073	0.081	0.069	0.065	0.071
DTX1	0.092	0.088	0.069	0.049	0.067	0.049	0.042	0.062
DTX2	0.177	0.065	0.080	0.073	0.048	0.070	0.048	0.066
PTX2	0.116	0.064	0.124	0.040	0.037	0.040	0.088	0.078
PTX11	0.252	-	0.043	0.040	0.037	0.040	0.088	0.078
AZA1	0.126	0.099	0.049	0.044	0.033	0.047	0.107	0.043
AZA2	0.183	0.117	-	-	-	-	-	-
AZA3	0.184	-	-	-	-	-	-	-
YTX	0.153	0.212	0.137	0.127	0.211	0.119	0.165	0.086
45 OH YTX	0.194	-	-	-	-	-	-	-
SPX1	0.084	0.051	0.053	0.037	0.032	0.068	0.084	0.053
GYM	0.034	0.057	0.038	0.042	0.052	0.062	0.044	0.040

Uncertainty in determination of recovery

The uncertainty in recovery of MLTs from each shellfish species was assessed using RSDs calculated from the recovery of toxins spiked into tissues at expected concentrations of 60 µg[OA]/kg, 100 µg/kg for DTX1, DTX2, PTX2, AZA1, SPX1 and GYM; and 250 µg[YTX]/kg (Table 16; section 3.2.6.). Outputs of standardised uncertainties are summarised in Table 22.

Table 22. Summary of standardised uncertainties associated with the determination of method recovery.

Toxin	Common mussel	Common cockle	Pacific oyster	Native oyster	King scallop	Queen scallop	Hard clam	Razor clam
OA	0.119	0.085	0.059	0.072	0.070	0.121	0.051	0.130
DTX1	0.059	0.097	0.093	0.065	0.054	0.096	0.069	0.126
DTX2	0.096	0.063	0.067	0.056	0.045	0.078	0.047	0.085
PTX2	0.059	0.103	0.042	0.028	0.023	0.034	0.047	0.030
PTX11	0.059	0.103	0.042	0.028	0.023	0.034	0.047	0.030
AZA1	0.048	0.047	0.035	0.039	0.016	0.023	0.036	0.029
AZA2	0.048	0.047	-	-	-	-	-	-
AZA3	0.048	-	-	-	-	-	-	-
YTX	0.050	0.074	0.111	0.134	0.096	0.110	0.079	0.173
SPX1	0.047	0.068	0.055	0.054	0.044	0.037	0.029	0.056
GYM	0.051	0.030	0.050	0.044	0.053	0.056	0.056	0.039

Contribution of *intra*-shellfish matrix effects

An assessment of recovery and its associated uncertainty cannot be made for every single sample. Instead, an assessment is made for sample classes, grouped by type of shellfish species (Anon. 2000). In terms of the variability associated with co-extracted matrix effects and its additional effect on measurement bias uncertainty, an assessment of these effects cannot be made for each individual sample without the use of quantitation by for instance, standard addition. In this context, such an approach would be impractical and costly for routine high throughput analysis. Nevertheless, such variability will potentially add an additional contribution to the overall measurement uncertainty of the method, although some judgement is required when inferring the degree of similarity within each class (*i.e.*, from shellfish species to each specific sample; Anon. 2000). Typically, the degree of similarity between *intra*-species samples is unknown, given the large amount of time and cost typically required to generate an appropriate level of data. It is for this reason that such matrix effects and their influences are often ignored when calculating overall measurement uncertainty. However, when suitable data are generated, then some attempt should be made to incorporate the *intra*-matrix variability into the overall contribution of measurement uncertainty.

Published guidance describes a number of approaches for incorporating uncorrected measurement bias into the overall uncertainty estimation (Donnell and Hibbert, 2005; Magnusson and Ellison, 2008). In the situation where the method bias is modest, a recommended approach proposed by Donnell and Hibbert (2005) is to incorporate the recovery correction (Δ) and the uncertainty of the correction (u^2_{Δ}) alongside the uncertainty of the uncorrected result (u_c) into the total combined standardised measurement uncertainty (U) as follows (Lira and Wöger, 1998):

Equation 2:

$$U = \sqrt{u_c^2 + \Delta^2 + u_\Delta^2}$$

In order to determine which of the above terms to include in the assessment, a significance test (two-tailed t-test, 95% confidence, *n*=8) was used to determine whether the mean recovery observed from the matrix variability study was significantly different from unity:

Equation 3:

$$t = \frac{|1-\overline{Rec}|}{u(\overline{Rec})}$$

where:

$\overline{Rec}$ = mean recovery, and

$u(\overline{Rec})$ = standard uncertainty calculated as the standard deviation of the mean (*i.e.*, RSD/√n) (Anon. 2000).

Evidence for an insignificant bias relating to the matrix effect would then remove the Δ^2 term from the above equation.

Investigations were carried out in order to assess the potential effects of matrix components in both Common mussel and Pacific oyster species from different spatial and temporal sources (see 3.2.4.). Results indicated a range of toxin recoveries within each species type and for each toxin, with each result expressed as a bias as compared with results obtained from analysis of solvent based standards. The variability of the bias determined was subsequently used to calculate the measurement uncertainty inherent in the matrix effects as expressed by the relative standard deviations of the bias for each toxin in both species. These results are summarised in Tables 23 and 24 for mussel and Pacific oyster tissues, respectively.

Table 23. Effects of *intra*-species variability of matrix effects on regulated and non-regulated toxin recovery from spiked Common mussel extracts collected over four month period with each sample analysed in replicate (*n*=5). Mean bias, standard deviations (s.d.) and *t*-test result (*t*-critical = 2.365; 95% confidence; *n*=8) are shown.

Mussel sample	Toxin							
	OA	DTX1	DTX2	PTX2	AZA1	YTX	SPX1	GYM
Original	0.87	1.04	0.90	0.99	1.12	1.3	0.81	0.98
Sample 1	1.01	1.22	1.08	1.27	1.65	0.9	1.80	1.35
Sample 2	1.20	1.40	1.20	1.09	1.11	1.4	1.09	1.01
Sample 3	1.25	1.06	0.96	0.82	0.76	1.3	0.85	0.82
Sample 4	1.19	1.29	1.20	1.01	1.00	2.4	1.16	0.97
Sample 5	1.05	1.30	1.13	1.02	1.02	1.4	0.95	0.98
Sample 6	0.99	1.10	0.91	0.86	0.97	1.1	0.99	0.86
Sample 7	1.14	1.15	1.05	0.98	1.03	1.4	1.15	1.01
mean	1.09	1.19	1.05	1.00	1.08	1.4	1.10	1.00
s.d.	0.13	0.13	0.12	0.14	0.26	0.46	0.31	0.16
RSD	11.9	10.8	11.5	13.7	23.6	33.1	28.2	15.6
s.d. of mean	0.042	0.038	0.041	0.048	0.084	0.117	0.100	0.055
t-test	2.051	5.101	1.331	0.080	0.964	3.436	0.982	0.059

Table 24. Effects of *intra*-species variability of matrix effects on regulated and non-regulated toxin recovery from spiked Pacific oyster extracts collected over four month period with each sample analysed in replicate (*n*=5). Mean bias, standard deviations (s.d.) and *t*-test result (*t*-critical = 2.365; 95% confidence; *n*=8) are shown.

Pacific Oyster sample	Toxin							
	OA	DTX1	DTX2	PTX2	AZA1	YTX	SPX1	GYM
Original	0.72	0.89	0.89	0.85	1.01	1.25	0.72	0.90
Sample 1	1.09	1.10	1.11	1.02	1.07	1.33	1.01	1.08
Sample 2	0.96	0.96	1.05	0.97	1.02	1.39	1.01	1.03
Sample 3	1.14	1.14	1.10	0.92	1.25	1.64	0.91	0.95
Sample 4	0.97	1.06	1.10	0.91	1.11	1.68	0.95	1.02
Sample 5	1.00	1.16	1.10	0.97	1.23	1.37	1.02	1.02
Sample 6	0.87	1.01	0.85	0.80	1.05	1.02	0.91	0.84
Sample 7	0.96	1.13	1.09	1.07	1.13	1.36	1.21	1.11
mean	0.96	1.06	1.04	0.94	1.11	1.38	0.97	0.99
s.d.	0.13	0.09	0.10	0.09	0.09	0.21	0.14	0.09
RSD	13.4	9.0	10.1	9.2	8.1	15.1	14.4	9.1
s.d. of mean	0.047	0.032	0.036	0.033	0.029	0.053	0.051	0.032
t-test	0.747	1.812	0.997	1.887	3.840	7.130	0.663	0.179

T-test results showed that the majority (75%) of toxins with a mean bias relating to matrix effects were not significantly different from unity. Consequently, the standardised uncertainties associated with the matrix effects were calculated from the uncertainties of the matrix-related bias (u^2_{Δ}) as expressed by the relative standard deviations of the mean bias and are summarised in Table 25.

Table 25. Summary of standardised uncertainties associated with co-extracted Common mussel and Pacific oyster matrix effects on the measurement of regulated and non-regulated MLTs.

Toxin	Common mussel	Pacific oyster
OA	0.119	0.134
DTX1	0.108	0.090
DTX2	0.115	0.101
PTX2	0.137	0.092
AZA1	0.236	0.081
YTX	0.331	0.151
SPX1	0.282	0.144
GYM	0.156	0.091

Combined measurement uncertainties

Preliminary, combined standardised uncertainties for each MLT in each shellfish matrix were then calculated from the square root of the sum of squares:

Equation 4:

$$u_c = \sqrt{u_1^2 + u_2^2 + u_3^2 + u_4^2}$$

where:

u_c = combined standardised uncertainty
u_1 = standardised uncertainties associated with precision
u_2 = standardised uncertainties associated with reproducibility
u_3 = standardised uncertainties associated with recovery determination
u_4 = standardised uncertainties associated with matrix effects (mussels and Pacific oysters only).

Currently, certified reference standards do not exist for PTX1, AZA2, AZA3 and 45 OH YTX. The uncertainty inherent in the precision associated with their determination by HPLC-MS/MS was therefore assumed to be identical to that of representative toxins of the three groups *i.e.,* PTX2, AZA1 and YTX, respectively. Standardised combined uncertainties were thus calculated and are summarised in Table 26. It is important to note that these data are preliminary. As more method performance data are obtained through routine implementation of the procedure and on-going analytical quality control, combined uncertainties are likely to change over time. Depending on the toxin/shellfish species combination, combined standardised uncertainties for individual toxins, ranged from 0.05 to 0.40. Mean values for standardised uncertainties for each species were between 0.10 and 0.26, with notably high values for the mussels, one of the species where the matrix effects are incorporated into the uncertainty budget. It may therefore be prudent for the determination of matrix effects to be extended to other species of interest validated in this study. However, it should be noted that the matrix influences exerted on toxins prepared in Pacific oyster extracts did not drastically increase the overall level of measurement uncertainty for this species.

Table 26. Summary of combined, standardised measurement uncertainties and their mean values (± one standard deviation; s.d.) calculated for the determination of regulated and non-regulated MLTs in test shellfish species.

Toxin	Common Mussel	Common cockles	Pacific oyster	Native oyster	King scallop	Queen scallop	Hard clam	Razor clam
OA	0.24	0.17	0.19	0.13	0.12	0.17	0.10	0.19
DTX1	0.21	0.16	0.17	0.14	0.10	0.14	0.12	0.19
DTX2	0.27	0.11	0.17	0.13	0.08	0.12	0.10	0.13
PTX2	0.21	0.15	0.17	0.06	0.05	0.08	0.12	0.11
PTX11	0.27	0.15	0.08	0.06	0.05	0.08	0.12	0.11
AZA1	0.28	0.12	0.12	0.09	0.05	0.06	0.12	0.08
AZA2	0.20	0.13	-	-	-	-	-	-
AZA3	0.21	-	-	-	-	-	-	-
YTX	0.40	0.24	0.26	0.22	0.25	0.22	0.21	0.23
45 OH YTX	0.26	-	-	-	-	-	-	-
SPX1	0.33	0.12	0.18	0.11	0.09	0.10	0.17	0.11
GYM	0.20	0.08	0.13	0.09	0.09	0.12	0.11	0.14
mean (± s.d.)	0.26 (± 0.06)	0.14 (± 0.04)	0.16 (± 0.05)	0.12 (± 0.05)	0.10 (± 0.06)	0.12 (± 0.05	0.13 (± 0.04)	0.14 (± 0.05)

Expanded measurement uncertainties were subsequently calculated after applying a coverage factor (k) of 2 in order to provide a 95% confidence in the distribution of values, assuming a normal distribution. Values are presented in Table 27 and for the regulated toxins, these ranged from 0.09 (AZA1/King scallop) and up to 0.80 (YTX/Common mussel). Excluding the non-regulated toxins SPX1 and GYM, mean values of expanded uncertainties for each species were between 0.20 and 0.51. The manner in which the individual expanded uncertainties are combined to yield a total measurement uncertainty based upon toxicity equivalent factors has not yet been decided. However, this does not detract from the relevance of the method performance data provided in this report.

Table 27. Expanded measurement uncertainties for the determination of regulated and non-regulated MLTs in test shellfish species.

Toxin	Common mussels	Common cockles	Pacific oyster	Native oyster	King scallop	Queen scallop	Hard clam	Razor clam
OA	0.48	0.34	0.38	0.26	0.24	0.35	0.20	0.38
DTX1	0.42	0.32	0.34	0.28	0.21	0.29	0.23	0.39
DTX2	0.54	0.21	0.34	0.26	0.16	0.24	0.20	0.27
PTX2	0.42	0.29	0.34	0.13	0.10	0.15	0.23	0.22
PTX11	0.55	0.29	0.17	0.13	0.10	0.15	0.23	0.22
AZA1	0.57	0.23	0.23	0.17	0.09	0.12	0.24	0.15
AZA2	0.41	0.26	-	-	-	-	-	-
AZA3	0.41	-	-	-	-	-	-	-
YTX	0.80	0.47	0.52	0.44	0.49	0.44	0.42	0.46
45 OH YTX	0.51	-	-	-	-	-	-	-
SPX1	0.65	0.24	0.36	0.22	0.18	0.21	0.34	0.21
GYM	0.40	0.15	0.27	0.18	0.18	0.24	0.23	0.27
mean (± s.d.)	0.51 (± 0.12)	0.30 (± 0.08)	0.33 (± 0.11)	0.24 (± 0.11)	0.20 (± 0.14)	0.25 (± 0.12)	0.25 (± 0.08)	0.30 (± 0.11)

3.3. Transfer of LC-MS/MS method and verification of method performance.

The HPLC-MS/MS method developed and refined on the Agilent *1100* HPLC and Waters *Quattro Micro* instruments was transferred to a second instrument [*Acquity* UPLC™ and *Xevo TQ* mass spectrometer (UPLC-MS/MS)] which was procured during mid-2010. Optimised mass spectrometric parameters were established for the production of precursor and two multiple reaction monitoring transition ions per analyte and these are reported in Appendix 25.

After converting the HPLC mobile phase gradient to UPLC, the detection of all regulated and two non-regulated toxins using positive and negative mode switching was complete within less than 8 mins. This is equivalent to a 65% reduction in the sample cycle time (injection-to-injection) as performed by the HPLC method. Comparisons of chromatograms of key lipophilic toxins after UPLC-and HPLC-MS/MS analyses are presented in Figures 13 and 14 showing the elution speed of the former method.

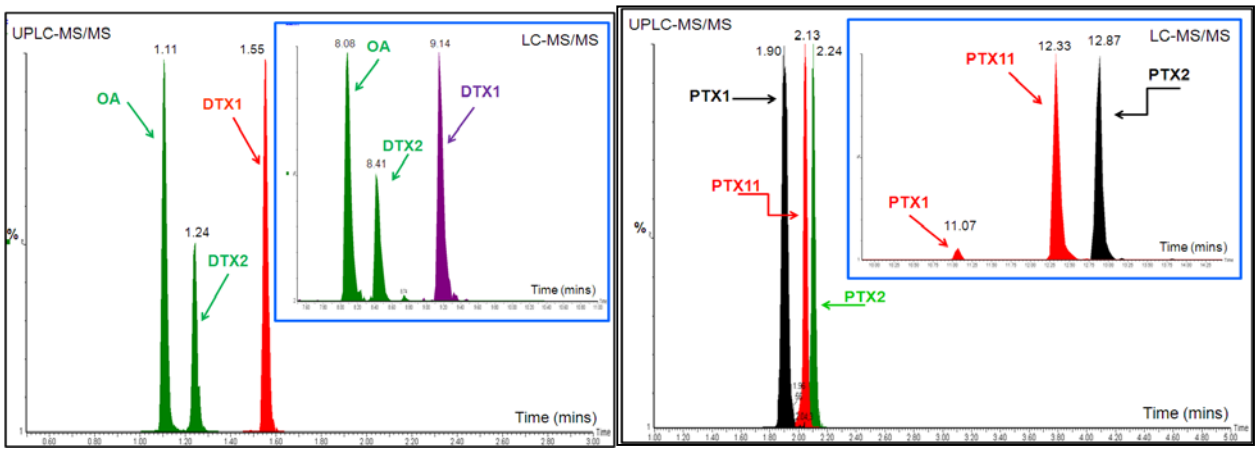


Figure 13. UPLC- and HPLC-MS/MS chromatograms of OA, DTX1/2 and PTX1/2/11 toxins.

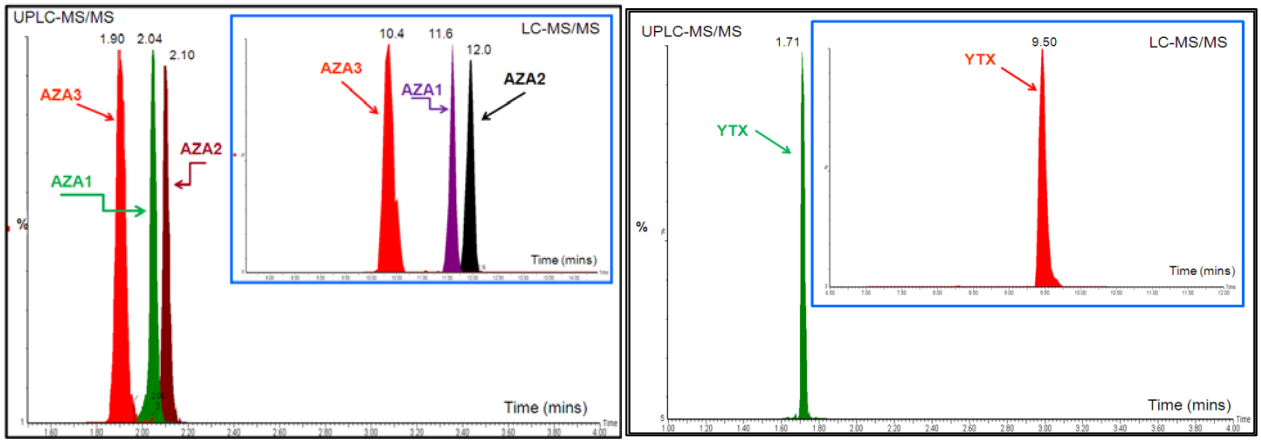


Figure 14. UPLC- and HPLC-MS/MS chromatograms of AZA1/2/3 and YTX toxins.

With an efficiency gain in the sample analysis time, this has a positive effect on sample throughput, and the UPLC-MS/MS method demonstrated its amenableness to processing high volumes of sample extracts. This was an important outcome from the analytical trials conducted on both instruments whereby daily batches of 20 shellfish samples were processed continuously over three and four weeks. The HPLC-MS/MS method was limited to an overnight run of 40 real shellfish extracts. These included 20 crude, methanolic extracts *plus* their hydrolysed extracts (for the analysis of DTX3s), together with calibration standards and a procedural blank. This number allowed laboratory analysts adequate data processing time throughout the following working day to compile and report toxin concentrations by the end of the day. As a consequence of the speed of analysis, an overnight sequence using UPLC is capable of processing up to 40 shellfish samples (*i.e.*, up to 80 extracts). Between the two trials, Cefas staff became experienced in improvements made to the operation of data transfer from the instrument's data processing software to a report-ready, Excel™ format. This, together with the speed of analysis demonstrated significant improvements in sample turnaround times. It was thus realised, that the UPLC-MS method was capable of meeting the considerable demands of lipophilic toxin surveillance that the Cefas laboratory occasionally experiences, particularly during the late spring, summer and early autumn months of a monitoring year.

Several method performance characteristics established during the in-house validation scheme involving the HPLC-MS/MS method were verified by deploying the UPLC-MS/MS instrument. Method selectivity, linearity of detection and limits of quantitation were investigated. Statistical comparisons of toxin concentrations derived from the analysis of naturally contaminated shellfish tissues using both instruments were also made.

3.3.1. Selectivity of the UPLC-MS/MS method.

The selectivity of the method was assessed by visual inspection of UPLC-MS/MS MRM chromatograms following analysis of toxin-free shellfish extracts from each of the species of interest. It was apparent that no interfering chromatographic peaks from co-extracted substances were found at, or close to the expected retention times of twelve regulated toxins as well as the two non-regulated analytes, SPX1 and GYM. The UPLC-MS/MS method showed acceptable selectivity.

3.3.2. Linearity of response of the *Xevo TQ* mass spectrometer.

Regressions constructed for the assessment of linearity of response on the Xevo *TQ* instrument indicated a good degree of linearity for the majority of toxins in methanol and shellfish extract, matrix matched standards. Evidence for this comes from the visual assessment of calibration plots and from calculated correlation coefficients (Table 28). Examples of calibration plots for AZA1 prepared in King scallop matrix extract and YTX in mussel matrix are provided in Figure 15 (a and b). Each plot shows the variation of peak area response per level of calibration.

Table 28. Linearity correlation coefficients (r^2) of the UPLC-MS/MS method.

Calibration matrix	Correlation coefficient (r^2)								
	OA	DTX1	DTX2	PTX2	PTX11	AZA1	YTX	SPX1	GYM
Methanol	0.999	0.997	0.997	0.999	0.999	0.999	0.999	0.999	0.999
Mussel	0.996	0.999	0.997	0.999	0.999	0.999	0.999	0.998	0.995
Cockle	0.997	0.999	0.999	0.998	0.999	0.999	0.999	0.999	0.999
Pacific oyster	0.998	0.999	0.998	0.998	0.998	0.999	0.998	0.997	0.999
Native oyster	0.997	0.995	0.999	0.999	0.999	0.999	0.999	0.999	0.999
King scallop	0.999	0.998	0.998	0.990	0.998	0.998	0.998	0.998	0.991
Queen scallop	0.998	0.999	0.998	0.999	0.998	0.999	0.999	0.996	0.998
Hard clam	0.998	0.999	0.998	0.990	0.989	0.998	0.998	0.992	0.994
Razor clam	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.998

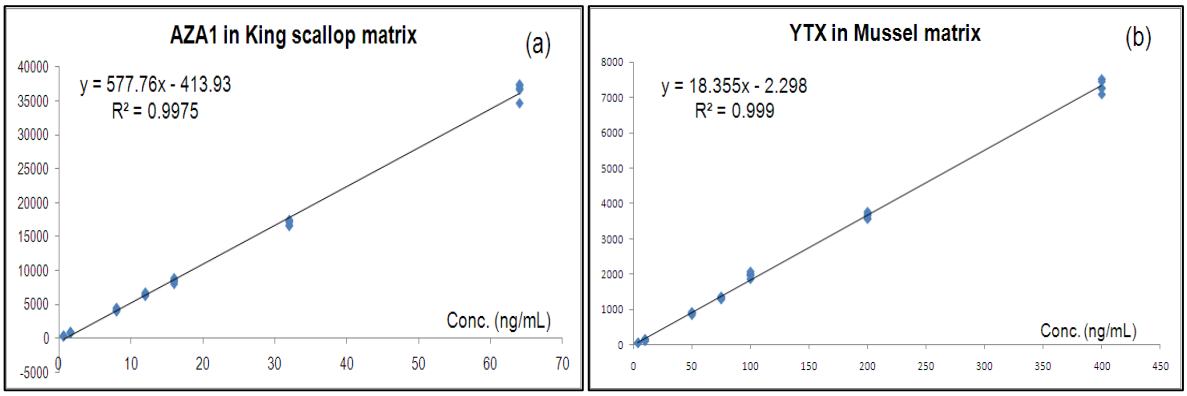


Figure 15. Calibration plots of (a) AZA1 in King scallop and (b) YTX in mussel matrix matched standards.

Due to the notable low variance of the replicate analyses at each concentration level, the F-test, lack-of-fit results showed many values higher than *F*-critical (Table 29). However, examination of the residuals of the regressions did not indicate any evidence for non-linearity in any of the plots as shown in Figures 16 and 17. It is noted that the variance in response arises from repeated instrumental injections, rather than replicated spiking experiments. The lower variability inherent in the instrumental precision of the *Xevo* system consequently results in the failed lack-of-fit results in many instances. For all toxins, use of non-linear regressions did not result in any improved regression fit including YTX, where on the *Xevo TQ* instrument linearity is evident up to 400 ng/mL. Linearity of this toxin as demonstrated by the *Quattro Micro* was limited to only 150 ng/mL. Consequently, the results indicated an acceptable linear relationship between concentration and detector response. Over the entire concentration range examined, this was apparent for all target analytes prepared in methanol solvent and in extracts of each of the shellfish matrices.

Table 29. Summary of *F*-test results from assessment of lack of fit on toxin calibrations generated on the *Xevo TQ* mass spectrometer instrument.

Calibration matrix	Toxin								
	OA	DTX1	DTX2	PTX2	PTX11	AZA1	YTX	SPX	GYM
Methanol	0.330	1.173	1.375	0.878	2.233	1.903	*4.181*	1.103	0.960
Mussel	*5.441*	0.454	*2.730*	0.912	*2.479*	1.066	*3.848*	*2.307*	*19.836*
Cockle	*4.388*	0.798	1.830	*7.599*	1.142	1.619	1.466	*2.469*	*11.607*
Pacific oyster	1.966	0.777	*2.504*	*20.273*	*10.354*	1.996	*9.929*	*23.054*	*11.612*
Native oyster	*3.119*	*10.034*	1.865	*4.733*	2.065	0.514	1.041	1.231	2.712
King scallop	*3.604*	*2.307*	*5.899*	*11.641*	*7.820*	*5.482*	*7.412*	*15.588*	*48.279*
Queen scallop	*3.390*	0.614	*10.216*	2.114	*14.569*	*3.111*	*6.397*	*44.878*	*8.579*
Hard clam	*4.037*	0.584	*6.505*	*57.264*	*82.964*	*3.681*	*13.339*	*50.129*	*27.829*
Razor clam	*6.316*	0.141	0.354	*6.846*	*6.727*	*2.921*	*13.396*	1.905	25.521

F-critical = 2.485 for all toxins. *F*-values > *f*-critical are highlighted.

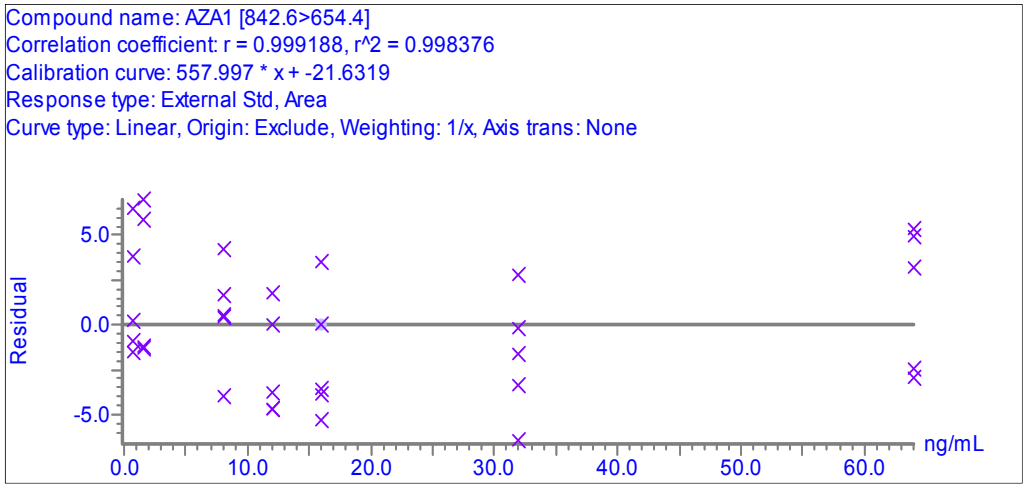


Figure 16. Percentage residuals of AZA1 in King scallop matrix matched calibration standards over the concentration range of 0.64 to 64 ng/mL.

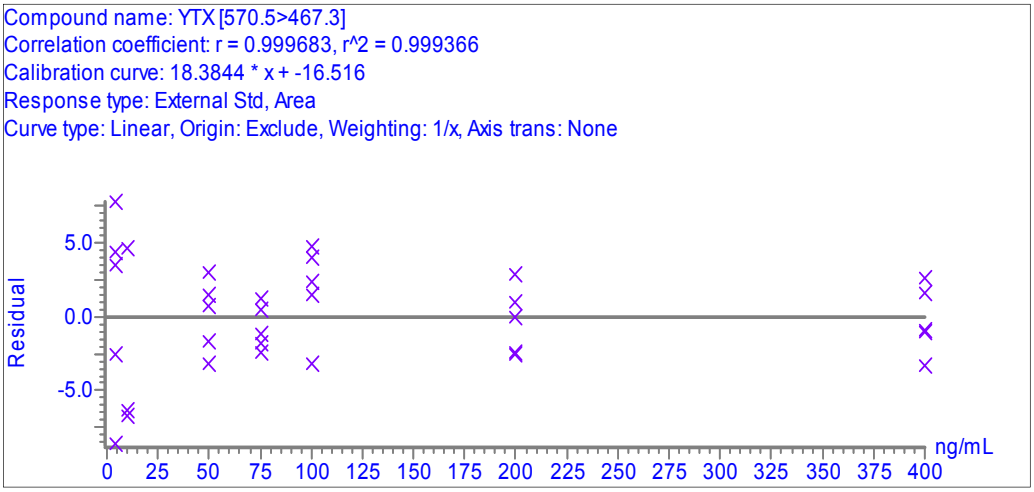


Figure 17. Percentage residuals of YTX in mussel matrix matched calibration standards over the concentration range 4 to 400 ng/mL.

In summary, the linearity of the UPLC-MS/MS method has been demonstrated for each of the target toxins in all of the matrices examined. Where appropriate, evidence for

a good linear relationship was obtained from a combination of visual examination of regressions and residuals, together with the assessment of correlation coefficients and lack-of-fit. The linearity was found to be acceptable in each matrix over the entire working range examined for each toxin. For the *Xevo* MS instrument, the linearity was shown for OA, DTX1, DTX2, PTX2, AZA1 and YTX over a range equivalent to 4 to 400% of the regulatory limits for each of the toxins. The linear range was also found to be acceptable for the non-regulated toxins SPX and GYM.

By calculating the ratio of matrix matched standard (MMS) calibration gradients to those obtained from methanol-based standard gradients provided information of matrix enhancement or suppression effects during UPLC-MS/MS analysis. Values close to unity indicate negligible effects, whereas ratios <1 and >1 describe influences due to suppression and enhancement effects. These ratios are presented in Table 30.

Table 30. Comparison of ratios of UPLC-MS/MS matrix matched calibration gradients to gradients from solvent based toxin calibrations.

Toxin	Shellfish matrix matched standard							
	Common mussel	Common cockle	Pacific oyster	Native oyster	King scallop	Queen scallop	Hard clam	Razor clam
OA	0.76	0.84	0.90	0.85	1.16	0.96	0.76	0.94
DTX1*	0.59	1.08	1.13	1.06	1.05	1.11	0.89	1.11
DTX2*	0.55	0.73	0.84	0.79	1.08	0.90	0.71	0.89
PTX2	0.47	0.66	0.61	0.66	0.77	0.64	0.62	0.61
PTX11**	0.55	0.66	0.80	0.80	0.66	0.65	0.81	0.53
AZA1	0.76	0.83	0.87	0.88	0.89	0.84	0.82	0.80
YTX	0.82	0.86	0.91	0.91	0.86	0.77	0.77	0.73
SPX1	0.47	0.63	0.60	0.64	0.73	0.61	0.58	0.67
GYM	0.53	0.64	0.71	0.73	0.75	0.68	0.58	0.65

***** Pre-certified reference standards ****** Non-certified reference standard

The response of OA was seen to be suppressed in the range of 4 to 24% when present in most shellfish extracts. The exception to this was the OA/King scallop combination where the analyte was mildly enhanced by 16%. Co-extracted matrices from cockle, oysters, scallops and Razor clam also exerted a weak-to-mild enhancement effect on DTX1. However, both DTX1 and DTX2 were found to be severely suppressed by >40% when prepared in the mussel extract. Again, King scallop matrix induced a mild enhancement on DTX2 whereas cockle, oysters, scallops and clams suppressed DTX2 responses in the range of 10 to 29%. For the pectenotoxins, all matrices suppressed their responses in the range of 19% (PTX11/Hard clam) to 53% (PTX2/mussel). Similar levels of suppression were apparent for the non-regulated SPX1 and GYM toxins. For AZA1, mild-to-strong suppression influences were seen. With the exception of the AZA1/mussel combination, effects were ≤20%. The response of YTX was also suppressed in the range of 9 to 27% for all matrices and Queen scallop and clam extracts produced the largest effects.

When applied to the analysis of OA/DTXs, PTXs and AZA1, the UPLC-MS/MS method appeared to be more susceptible to suppression influences derived from mussel tissue than from other shellfish matrices. A complexity of suppression and enhancement effects were exhibited by the HPLC-MS/MS method using the *Quattro Micro*. It was difficult to identify common effects between the two instruments. Other than, where YTX was seen to be enhanced during analysis using the *Quattro Micro* with effects reaching over 50%, this toxin was found to be suppressed during UPLC-MS/MS analysis. Matrix effects have been evaluated by Fux *et al.* (2008) using acidic mobile phase gradients,

HPLC-MS/MS (with a C_8 LC column) and UPLC-MS/MS using BEH C_8 and C_{18} columns. In the published work involving crude mussel MMS (solvent-to-sample ratio of 10:1) containing OA, PTX2 and AZA1, OA was seen to be enhanced in the range of 19 to 50%. Where PTX2 was weakly suppressed using UPLC and the BEH C_8 column, it was strongly enhanced by 45% with HPLC (C_8) and by 93% using UPLC (BEH C_{18}). Negligible-to-weak suppressions were exerted on AZA1 using C_8 columns and both HPLC and UPLC; with a C_{18} column and UPLC, this toxin was also strongly suppressed by 34%. Observations described by These *et al*. (2009) indicated that using a mildly basic (pH 7-8) LC mobile phase gradient, OA was found to be enhanced whereas PTX2 and AZA1showed evidence of signal suppression. By contrast, YTX was not significantly influenced.

From the findings of the in-house validation scheme presented in this report and from evaluations published by other workers, it is readily apparent that contrasting matrix influences on MLT determinations exist. These are most likely due to the differences in mass spectrometric instrumentation used for the ionisation of MLTs and in particular, differences associated with the ion source geometries. Other confounding factors include the chemical nature of shellfish extracts, matrix differences between shellfish of the same and different species, stationary phase chemistries of LC columns and of mobile phases applied to analyte separation. Furthermore, the application of HPLC- and UPLC-MS/MS using the same (BEH) column stationary phase and mobile phase chemistries has resulted in differences of matrix effects as it has been demonstrated during the in-house validation work reported here. Most probably, these inconsistencies arose due to chromatographic separation differences rather than MS source geometries since both instruments were equipped with the same z-spray-type ionisation source. However, it is necessary to control matrix effects and further work is recommended. Evidence of improving the quality of analytical data from the application matrix correction is presented later in section 3.4.3.

3.3.3. Estimated instrumental limits of quantitation of the UPLC-MS/MS method.

Limits of quantitation (LOQ) of the UPLC-MS/MS method were derived from replicated injections of shellfish extracts fortified at concentrations equivalent to entire (extraction *plus* analytical) method LOQ values as determined by the HPLC-MS/MS method (Table 14; section 3.2.5.). By applying a peak height signal:noise ratio of 10:1, LOQs were estimated from measured concentrations (Table 31).

Table 31. Estimated instrumental limits of quantitation of the UPLC-MS/MS method.

Shellfish matrix	Estimated LOQ (µg/kg)								
	OA	DTX1	DTX2	PTX2	PTX11	AZA1	YTX	SPX1	GYM
Mussel	8.7	3.6	5.3	2.3	2.0	1.9	15	3.3	3.1
Cockle	9.4	10	6.5	2.0	1.7	7.2	9.5	1.2	2.1
Pacific oyster	9.2	5.8	6.3	1.9	2.0	1.3	6.9	1.2	3.1
Native oyster	8.7	9.6	8.8	3.2	2.4	2.5	16	1.0	2.3
King scallop	7.9	6.2	8.3	2.2	3.4	1.9	10	0.8	5.4
Queen scallop	9.7	4.3	7.0	3.0	3.2	2.5	8.1	0.4	4.8
Hard clam	8.4	6.3	8.9	2.7	2.1	6.8	13	1.1	3.1
Razor clam	6.5	8.8	5.5	4.0	2.1	1.8	9.3	0.6	3.6

For the OA/DTX group, LOQs were ≤10 µg/kg and respectively, average OA, DTX1 and DTX2 values were 9, 6 and 7 µg/kg. Levels of LOQs for this toxin group and achieved by the HPLC-MS/MS method were considerably higher and in the range of 23-58 µg/kg. Mean LOQs estimated for PTX2 and PTX11 were similar and ~2.5 µg/kg. This was almost three times lower compared to the average PTX2 LOQ determined by the older instrument using conventional HPLC separation. When the ranges of LOQs for AZA1 were

compared, little difference was found between the performances of the two instruments. Upper LOQ for both HPLC- and UPLC-MS/MS methods were ~7.5 µg[AZA1]/kg. Where LOQs were between 28 and 105 µg/kg for YTX and obtained from the *Quattro Micro*, the range of values estimated from the UPLC-MS/MS was 8-16 µg/kg. For the non-regulated SPX1 and GYM toxins, LOQs for the former toxin and achieved by both instruments were between 1 and 2 µg/kg. For the latter analyte, an average LOQ level over three times lower to that determined from the *Quattro Micro* was apparent.

Where the analysis of the free OA and DTX toxins is concerned, the HPLC-MS/MS method was found to be relatively insensitive. However and with the application of UPLC-MS/MS, lower LOQs of ~6% of the regulatory limit (RL) can be attained for each of these toxins. When toxicity equivalent factors are applied to averaged OA and DTX LOQs, summed LOQ values are 12% of the 160 µg[OA eq.]/kg limit. The HPLC-MS/MS method was only capable of achieving 52 to 76% of this limit. Sensitivity improvements have been gained by the investment made by Cefas in modern LC and MS technology, and this is particularly evident for the determination of the classical diarrhetic shellfish poisoning toxins.

3.3.4. Statistical comparison of toxin concentrations determined from two LC-MS/MS instruments.

Concentration data obtained from the determination of MLTs in a selection of naturally contaminated mussel, oyster and cockle samples and derived from HPLC- and UPLC-MS/MS methods are summarised in Table 32. The samples contained a range of concentrations of OA, DTX and AZA toxins. For the purposes of determination of equivalence in instrumental performance, data points were only included where toxin concentrations were within the linear working range of the two instruments. Correlations of concentration data derived from both instruments are displayed in Figure 18.

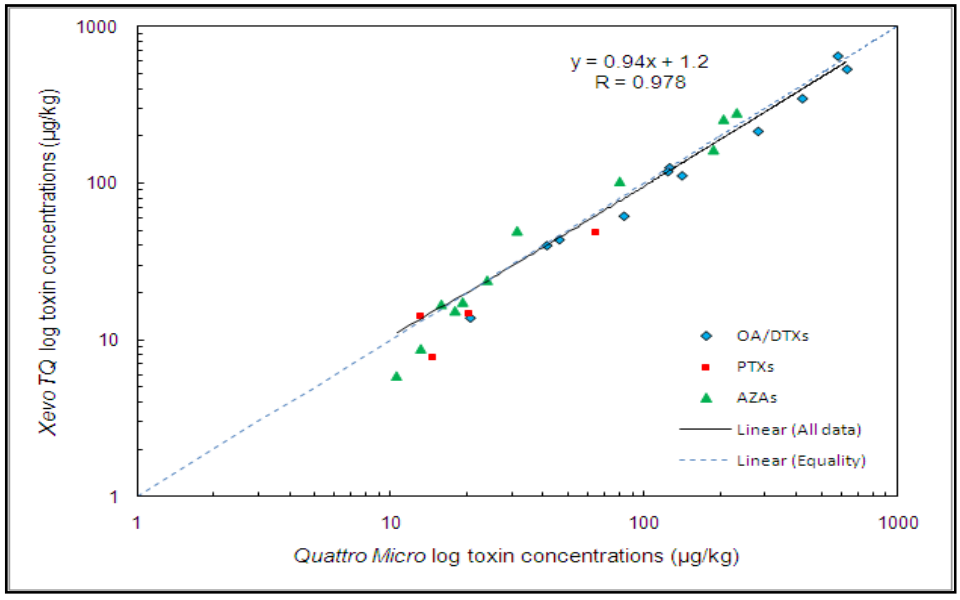


Figure 18. Comparison of log transformed toxin concentrations (µg/kg) determined in naturally contaminated shellfish using the the *Quattro Micro* and *Xevo TQ* LC-MS/MS instruments, showing 95% confidence limits and equality.

Results for all of the combined samples showed a satisfactory overall agreement between concentrations determined from the two instruments. A good correlation between

the two data sets was seen with a Pearson correlation (r) equal to 0.978 and the correlation gradient close to equality. A *t*-test (paired two sample for means) performed on the two sets of toxin concentrations resulted in a *t*-value of 0.933 and a p-value of 0.360 (two-tailed, 95% confidence; *t*-critical=2.060; *n*=26). This indicated that the average difference in the two data sets was not shown to differ significantly from zero. Whilst the difference in values was effectively zero, the correlation regression equation does show a potential relationship between the difference in values returned and the toxin concentrations determined. This is examined further in Figure 19(a) with a Bland-Altman plot, specifically highlighting a slight effect of toxin concentration on the differences observed, as shown by the trend in value. A log plot is utilised due to the wide range of toxin concentrations incorporated and mean difference and agreement limits [±2 standard deviations (s.d.)] are also displayed.

Table 32. Comparison of mean toxin concentrations [± one standard deviation (s.d.)] and between batch percentage relative standard deviations (RSD_R) determined from the analysis of naturally contaminated shellfish tissues using two LC-MS/MS instruments.

Toxin	Shellfish sample	HPLC-MS/MS		UPLC-MS/MS	
		mean conc. ± one s.d. [µg/kg] (*n*)	RSD_R (%)	mean conc. ± one s.d. [µg/kg] (*n*)	RSD_R (%)
Free OA	Mussel	41.4 ± 7.70 (*5*)	18.6	39.9 ± 4.63 (*5*)	11.6
	Mussel	126 ± 10.1 (*5*)	8.0	125 ± 16.0 (*7*)	12.8
	Mussel	629 ± 75.5 (*7*)	12.0	529 ± 65.1 (*5*)	12.3
Total OA	P. oyster	83.3 ± 9.8 (*7*)	11.7	61.4 ± 6.14 (*5*)	10.0
	Mussel	124 ± 19.0 (*7*)	15.3	118 ± 11.8 (*7*)	10.0
	Mussel	141 ± 20.9 (*5*)	14.8	111 ± 10.9 (*5*)	9.8
	Mussel	281 ± 47.8 (*5*)	17.0	213 ± 36.2 (*5*)	17.7
Free DTX1	Mussel	579 ± 48.6 (*7*)	8.4	642 ± 79.0 (*5*)	12.3
Total DTX1	P. oyster	20.7 ± 5.20 (*4*)	15.1	13.8 ± 1.89 (*6*)	13.7
	Mussel	420 ± 60.5 (*7*)	14.4	344 ± 56.7 (*4*)	16.5
Free DTX2	Mussel	46.3 ± 10.3 (*7*)	22.3	43.5 ± 5.48 (*5*)	12.6
PTX1	Mussel	13.0 ± 4.28 (*7*)	32.9	14.1 ± 4.78 (*5*)	33.9
PTX2	Mussel	14.6 ± 2.35 (*5*)	16.1	7.70 ± 1.05 (*8*)	13.6
	P. oyster	20.3 ± 3.45 (*7*)	17.0	14.9 ± 1.30 (*9*)	8.7
	Mussel	64.4 ± 5.73 (*7*)	8.9	48.3 ± 3.57 (*9*)	7.4
AZA1	P. oyster	13.2 ± 1.74 (*5*)	13.2	8.80 ± 1.59 (*3*)	18.1
	Mussel	19.3 ± 3.88 (*5*)	20.1	17.5 ± 1.68 (*8*)	9.6
	Mussel	24.1 ± 1.69 (*7*)	7.0	24.1 ± 2.34 (*5*)	9.7
	Cockle	205 ± 27.3 (*5*)	13.3	257 ± 20.3 (*6*)	7.9
AZA2	Mussel	15.9 ± 2.91 (*3*)	18.3	16.9 ± 2.69 (*7*)	15.9
	P. oyster	18.0 ± 3.26 (*5*)	18.1	15.4 ± 1.80 (*3*)	11.7
	Mussel	31.6 ± 7.78 (*7*)	14.6	49.8 ± 4.03 (*5*)	8.1
	Cockle	187 ± 14.4 (*5*)	16.5	164 ± 14.6 (*6*)	8.9
AZA3	Mussel	10.6 ± 1.81 (*5*)	17.1	5.9 ± 0.713 (*5*)	12.1
	Mussel	80.0 ± 12.1 (*7*)	15.1	103 ± 14.5 (*3*)	14.1
	Mussel	231 ± 31.0 (*7*)	13.4	282 ± 20.0 (*6*)	7.1

n = number of replicate shellfish samples extracted and analysed.

Specifically, the results indicated some evidence for higher results determined by the *Quattro Micro* instrument at lower toxin concentrations. It is possible that at lower concentrations, the lower relative sensitivity of the older *Quattro Micro* mass spectrometer may result in a relatively larger addition of background chromatographic noise to the toxin peak signal. Certainly, with removal of samples exhibiting toxin concentrations <20 µg/kg (equivalent to 12.5% of the regulatory limit for those toxins presented in Table 32), this apparent trend is removed as demonstrated in Figure 19(b). There is also the potential for differences in the response of the two instruments due to exerted co-extracted matrix effects during source ionisation. However, without further analysis of a larger number of contaminated shellfish samples over the entire linear working range of the two instruments, it is currently not possible to conclude what the exact causes may be for such a deviation.

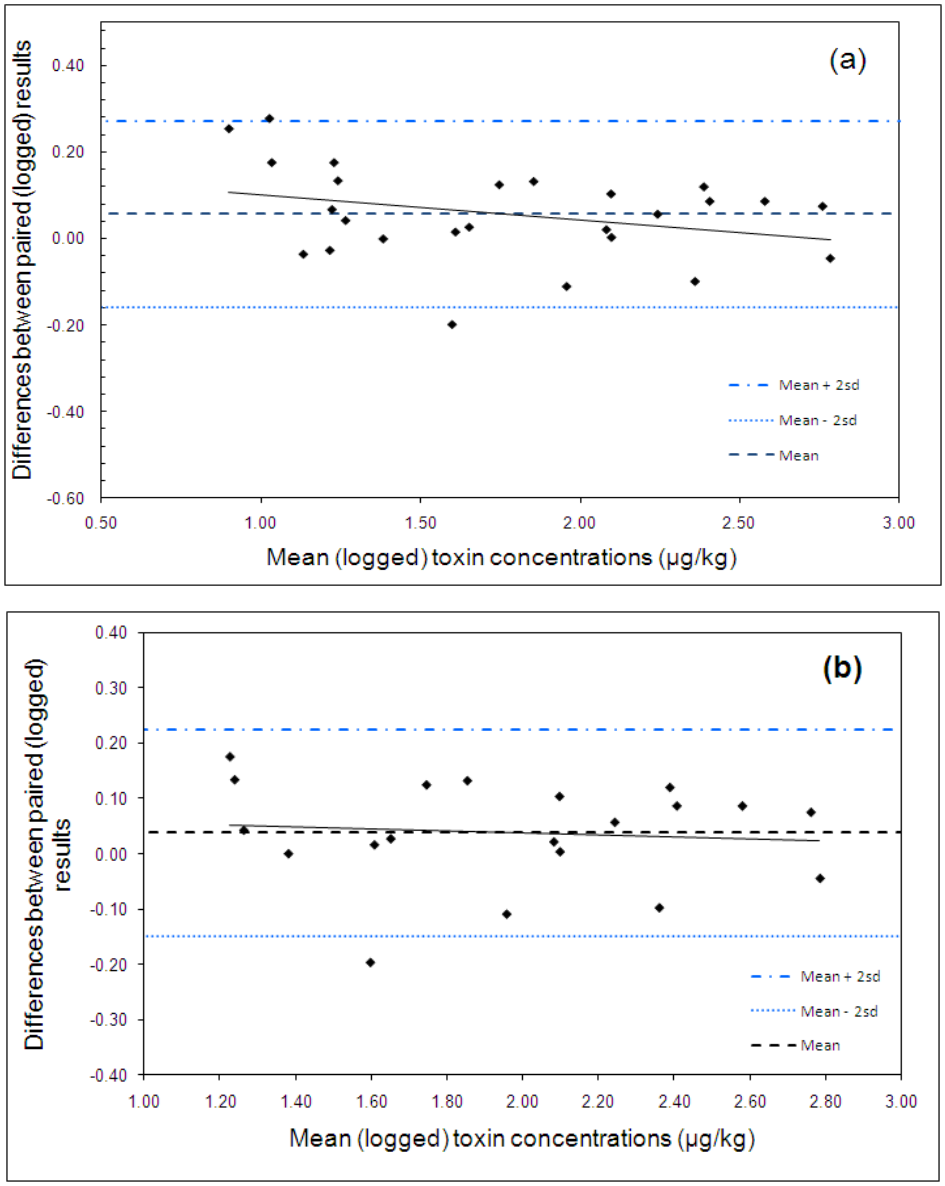


Figure 19. Log plot of differences in paired toxin concentrations against mean toxin concentrations (µg/kg), highlighting mean difference [d] and agreement limits [d±2 standard deviations; s.d.], for a) all samples b) concentrations of <20 µg/kg removed.

The results have demonstrated a good, general agreement between the MLT concentrations derived by the two LC-MS/MS systems currently in use at Cefas. There was no statistical difference between the average results returned and only a slight trend evident in the relationship between results differences and toxin concentration. This may to relate to the relative effects of background noise at concentrations well below the regulatory limit or matrix influences on analyte ionisation. It is evident that the performance of the two instruments in terms of the quantifying OA, DTXs, PTXs and AZA toxins concentrations is comparable.

3.4. Performance of the HPLC-MS/MS method in interlaboratory studies.

3.4.1. Performance of HPLC-MS/MS method within the QUASIMEME programme.

The HPLC-MS/MS method was applied to the determination of OA/DTX and AZA toxins in standard solutions, shellfish extracts and tissue samples provided under the QUASIMEME performance study programme (section 2.7.1.). Free and total (*i.e*. free concentrations *plus* esterified) contents of OA and DTX toxins were obtained after sample hydrolysis. Cefas concentrations, assigned means and the *z*-score values describing the performance of the HPLC-MS/MS method are presented in Appendices 26 to 28. For the majority of the test samples, *z*-scores indicated satisfactory ($|z| \leq 2$) performance of the method. However, several determinations were classified as questionable ($|z|$-score from 2 to 3) and unsatisfactory ($|z| \geq 3$). For those unsatisfactory results, these related to total OA concentrations being determined beyond the upper limit of working linear range. This was particularly apparent for round 59 (Appendix 27) and for the quantitation of total OA in mussel and King scallop tissues. Respectively, total OA concentrations were 33 and 41% higher than assigned values. These overestimations may have been due to OA signal enhancement effects associated with the hydrolysed extracts. However in contrast, it was observed that extracts from a mussel and a K. scallop sample suppressed OA by ≥10% (Table 13; section 3.2.3) although extracts were unhydrolysed. As a quality control procedure, it will be necessary to dilute sample extracts showing MLT concentrations outside the upper extreme of the working calibration range and re-analyse to ensure values fall within this range. For most of the questionable results, these were due to reporting concentrations at approximately their limits of detection or close to their limits of quantitation (section 3.2.5; Table 14).

Interestingly for the AZA analogues, Cefas laboratory means were consistently lower than the assigned values. This was apparent for AZA3 (Appendix 27) and AZA2 and AZA3 (Appendix 28). In comparison to assigned, mean concentrations, laboratory AZA2/3 values were between 23 and 36% lower for the mussel samples (numbers 91 and 92; Appendix 28). Since AZA2 and AZA3 were unavailable in certified reference format at the time of undertaking this exercise, the calibration plot derived from the AZA1 reference standard was used indirectly to measure their concentrations. With reference to Figure 6 (section 3.2.2.), AZA1 and AZA3 responses were found to be similar when prepared in methanol solvent. Thus the assumption can be made that by applying AZA1 calibrations, the quantitation of AZA3 would likely to be unbiased. It should be noted that response plots shown in Figure 6 were prepared from the analysis of a pre-release reference solution containing AZA1/2/3. Since the concentrations provided by IMB (Canada) were not certified, it is possible that trends shown by the gradients of calibration plots may not be entirely accurate. Once AZA2 and AZA3 are released as certified reference standards and then combined with AZA1, response factors can then be accurately determined. For both mussel extracts, AZA3 was 30-36% lower than assigned values. It was possible that suppression influences during analyte ionisation and derived from the extracts may have

resulted in this finding. However, only a 10% suppression effect was determined for AZA3 in a mussel extract during in-house validation (Table 13; section 3.2.3.).

For sample 91 and in comparison to the assigned value, AZA1 was also lower in concentration reflecting a suppression effect associated with the HPLC-MS/MS method. In contrast and for sample 92, laboratory and assigned AZA1 values only differed by 7%. According to AZA analogue responses (Figure 6), AZA2 concentrations should be overestimated when applying indirect (from AZA1 calibration) quantitation. Laboratory AZA2 concentrations were also lower than assigned values. Again, this may have been due to strong suppression effects associated with these particular test samples. This however, contradicts the 12 and 15% enhancement effects exerted on AZA2 and AZA1 (Table 13). As shown in Figure 8 (section 3.2.4), a 65% enhancement effect was also measured for AZA1 in a mussel sample taken from Swansea (Wales; March 2010). Contrastingly, a 25% suppression effect was evident for the same toxin determined in a different mussel sample taken from Lewis and Harris (Scotland; April 2010). Evidence from AZA concentration data generated in this proficiency exercise would support findings described in 3.2.3 and imply that matrix influences on AZAs as well as OA/DTXs differ on an *intra*-species basis. In relation to the application of the HPLC-MS/MS method under validation, there is a need to apply direct quantitation to the determination of these toxins. It thus stresses the need to have AZA2 and AZA3 certified reference standards available to address the potential for bias of determination and a strategy for overcoming matrix influences during analysis.

3.4.2. Performance of the HPLC-MS/MS method in the BVL (Germany) collaborative study.

For the BVL main collaborative study (2009), the HPLC-MS/MS method twas applied to the determination of OA/DTX, PTX, AZA and YTX toxins in cooked mussel tissues and extracts as well as in extracts from uncooked and spiked oyster and clam tissues (section 2.7.2.). Each of the three analytical sequences met the quality control criteria (linear regression coefficients ≥0.98 and response drift between two calibration plots ≤25%). In terms of toxin recovery from spiked extracts, the method performed satisfactory. Recoveries ranged from 95 to 103% for OA, PTX2, AZA1 and YTX (Table 33).

Table 33. Percentage toxin recoveries obtain from the HPLC-MS/MS analysis of fortified shellfish extracts (BVL main collaborative study, July-September 2009).

Sample no.	Sample description	Recovery (%)			
		OA	PTX2	AZA1	YTX
5	Cooked mussel extract		100		
6	Oyster extract	98	102	106	97
7	Clam extract	100	95	103	95
8	Oyster extract	100	100	94	100

To assess the performance of individual methods, each participant was provided with *z*-scores in the form of a toxin/sample graphical plot (Appendix 29). Scores were provided where the laboratory's measurement values were above the limit of detection. The overall performance of the Cefas method was seen to be satisfactory and all scores were between -2 to 1.3 (Table 34). The HorRat values of analytes for which certified standards were available (AZA1, OA, YTX and PTX2) ranged between 0.6 and 1.5 and demonstrate applicability and robustness of LC-MS/MS as a method for MLT measurements. HorRat values of indirectly quantified analytes for which no certified

standards were available (*i.e*., AZA2, AZA3, DTX1, DTX2, 45 OH YTX) were between 0.7 and 2.07, and the majority were <1.6.

Conclusions drawn by the interlaboratory study coordinators regarding correction for matrix influences on toxins from spiked extracts indicated that this resulted in only a marginal increase (from 93% to 96%) of the mean recovery of all toxin analytes (Uhlig *et al*., 2010). Under method repeatability (*i.e*., within batch analysis) conditions, matrix correction had a minor effect on concentration data. However, it was observed that matrix correction had a pronounced impact in terms of reducing systematic variations between batch analyses conducted within a laboratory, as well as reducing those variations between different laboratories. Based on these observations, it was recommended that matrix correction is applied during LC-MS/MS analysis. Recently and from the peer reviewed publication of the BVL study, it was been concluded by These *et al*. (2010) that LC-MS/MS, as a tool for determining MLTs in shellfish, is satisfactorily robust even when different laboratories apply different LC-MS/MS methods.

Table 34. Approximate Cefas *z*-scores obtained for the analysis of MLTs in cooked mussel tissues and extract, and in uncooked oyster and clam extracts. (See Appendix 29 for graphical plots of *z*-scores).

Sample no.	Sample description	*z*-score								
		OA	DTX1	DTX2	PTX2	AZA1	AZA2	AZA3	YTX	45 OH YTX
1	Cooked mussel	0.2		-0.2		0.1	0.5	-1.2	-	
3	Cooked mussel	1.0		-0.4		0.2	0.5	-1.1	-	
4	Cooked mussel	-0.2	0.0	-0.3		-0.2		0.3	0.2	0.9
5	Cooked mussel extract (spiked)	0.1		-0.3	-0.2	-0.2	0.1	1.3	-	
6	Oyster extract (spiked)	0.1			0.2	0.2			1.0	
7	Clam extract (spiked)	0.0			-2.0	-0.5			0.9	
8	Oyster extract (spiked)	0.1			-0.3	0.3			1.3	

3.4.3. Performance of the HPLC-MS/MS method in the RIKILT (Netherlands) collaborative study.

Cefas' participation in this study was of particular relevance since all laboratories were required to apply the alkaline LC gradient that we had adopted and refined for the purposes of the in-house validation scheme. With reference to Appendices 30 to 33, Cefas laboratory mean toxin concentrations and assigned means are provided for each of the eight test shellfish samples involving mussels (*n*=5), oysters (*n*=2) and a cockle. All data reported by the coordinators were not corrected for recovery losses. Since participants were required to use mussel matrix matched standards (MMS) to generate toxin calibration plots, concentration data are presented as matrix corrected. Within and between laboratory relative standard deviations and HorRat values are also presented. Concentrations of DTX1 and DTX2, and AZA2 and AZA3 have been reported from direct determinations using DTX1 and DTX2, and AZA2 and AZA3 calibration plots, and from indirect quantitation using OA and AZA1 as representatives of the two toxin groups.

Based on comparing the Cefas laboratory mean concentrations for target analytes with assigned means, the following observations were made with respect to the performance of the HPLC-MS/MS method under validation:

- the quality control criteria ($r^2 \geq 0.98$ and response drift ≤25%) were achieved for free OA and DTX2, free and total DTX1, AZA1, AZA2 and AZA3, and YTX calibrations. For total OA and total DTX2 calibrations, response drift values were respectively, 33.2 and 25.9% and outside the criteria. On this basis, Cefas concentration data were not accepted for statistical analyses. However, concentration data have been presented here for purposes of assessing the performance of the analytical method
- for the OA/DTX toxin group, and from their determination using the mussel matrix standard (MMS) approach, quantities of free OA were found to be consistently ~1.2 times higher than assigned mean values. However, for the measurement of total OA (free *plus* esterified OA as determined after extract hydrolysis), laboratory and assigned mean values were similar
- by applying direct DTX1 and DTX2 calibrations to the quantitation of both free and total DTX1 and DTX2 showed that the Cefas mean values were similar to the assigned mean data. However, where free DTX1 and DTX2 were determined indirectly from OA calibrations, laboratory means were higher than assigned means by respective factors of ~1.5 and ~1.2. Total DTX1 measured indirectly were also higher by ~1.6. It was interesting to note from the coordinators remarks that for six participants, free DTX1 concentrations, derived directly from DTX1 calibrations, were ~1.2 times greater than values obtained from OA calibrations (van den Top, 2010). The performance of the Cefas HPLC-MS/MS method was found to be the opposite of this phenomenon. Most likely, this reflects differences of ionisation responses of OA and DTX1 compared to responses obtained by LC-MS/MS instrumentation used by the other participants
- in terms of method reproducibility, RSD_R values were between 12.0 and 26.1% for the measurement of free DTX1 when this analyte was quantified directly from DTX1 calibrations. This range was considerably lower compared to values (27.7-49.4%) when OA calibration was used. Similar observations were found for total DTX1
- PTX2 was only detected in PTX2-spiked mussel and oyster tissues. Both Cefas laboratory means and assigned means were similar. Recoveries of PTX2 obtained by the Cefas method were good and ≥90%. Additionally, the method was capable of detecting low levels of PTX2 in samples 5 (mussel) and 7 (cockle) at ~10 and 15 µg/kg, respectively
- for the AZA1/2/3 toxins, both Cefas and assigned means were similar. There was little difference between laboratory and assigned means when AZA2 was determined either directly from the AZA2 calibration plot or indirectly from AZA1 calibrations. Between laboratory RSD_R and HorRat values were also similar and acceptable when both direct (7.2-20.0%; HorRat 0.27-0.68) and indirect AZA2 (10.3-14.4; HorRat 0.37-0.58) quantitation was performed. This suggest that AZA2 can be quantified using AZA1 MMS calibrations
- However, when comparisons are made between Cefas mean concentrations for AZA3 determined by direct (AZA3) and indirect (AZA1) quantitation, values obtained by the direct approach were higher by a factor of ~1.5. The same is reflected when only assigned means are compared. Although HorRat values calculated for both direct and indirect quantitation approaches were all <0.85, method reproducibility was seen to be better when AZA3 direct quantitation was applied
- for YTX analogues, YTX and 45 OH YTX were quantified in test samples. Only Cefas laboratory YTX mean concentrations for mussel samples 1 and 5, and the cockle sample were similar to the assigned values. Means from the two other mussels samples (2 and 3) were higher by factors of 1.9 and 1.3, respectively. The

same was apparent for the measurement of 45 OH YTX in these samples. However, the opposite was evident for mussel sample 6 where the laboratory mean was less than the assigned value. It would appear that the Cefas method did not perform so well for the determination of YTXs. It may be possible that the application of matrix correction was not as effective for this toxin as it has been shown for other analytes such as DTX1 and DTX2. This may have influenced final YTX concentrations. Where participants were capable of detecting YTX at ~40 µg/kg in the Pacific oyster sample, the Cefas HPLC-MS/MS method was unable to achieve this detection as it was below the LOD for this matrix. Method reproducibility for the indirect determination of 45 OH YTX by YTX calibration was shown to be high as reflected by RSD_R (27.3-40.5%) and HorRat values (≥1.5)

From this study, the determination of MLTs was performed via the application of a mussel MMS approach. Thus, there was a lack of information regarding the degree of matrix influences that may have been apparent if calibration solutions had been prepared in methanol solvent. The conclusion published by Gerssen *et al.*, (2010) from the single laboratory validation of the alkaline LC gradient stated the use of MMS largely eliminates ion suppression and enhancement effects. Furthermore, it was shown that MMS using blank mussel extracts can be used to quantify toxins in other shellfish matrices such as oyster, cockle and clam. Species differences did not have a significant effect on the analytical method. Where comparisons between Cefas' laboratory means and the assigned mean concentrations were made from the RIKILT study, and particularly for the determination of OA/DTXs, PTXs and AZA toxins, the outcome of these indicate that the performance of the Cefas LC-MS/MS method supported the conclusions described by Gerssen *et al.,* (2010). However, further work involving both of the Cefas LC-MS/MS instruments would be required to verify these observations.

3.4.4. Performance of the HPLC-MS/MS method in the EU-RL (Spain) collaborative study.

With reference to Appendices 34 to 41, Cefas laboratory mean toxin concentrations and assigned means have been provided for each of the seven test shellfish samples involving uncooked mussel (*n*=1), cooked mussel (*n*=2), three clam species (Razor, Wedge shell and Striped Venus) and a cockle. Levels of DTX1 and DTX2, and AZA2 and AZA3 have been reported from (1) direct determinations using DTX1 and DTX2, and AZA2 and AZA3 calibration plots (prepared from a multi-toxin standard solution), and from (2) indirect quantitation using commercially available, OA and AZAZ1 certified reference standards. The information has been summarised in Table 35 for OA and DTXs, and Table 36 for PTX2, AZAs and YTX analytes. At the time of writing, only total OA and DTX concentrations (as determined after extract hydrolysis) had been provided by the study coordinators.

All data provided by the European Reference Laboratory for Marine Biotoxins draft report (EU-RL, 2010) have not been corrected for recovery losses. As well as presenting uncorrected concentrations, concentrations for all toxins are also provided here after matrix correction using a mussel matrix matched standard (MMS). For OA and DTX toxins only, an extract from the IMB NRC Canada Mus-b certified reference material was also used by the participants to matrix-correct OA/DTX concentrations. With regards to those shellfish species applicable to Cefas' in-house method validation (*i.e*., uncooked mussel, cockle and razor clam), a review of the findings from this collaborative study has been based on:

1. the performance of the Cefas HPLC-MS/MS method in terms of describing the degree of matrix influences by comparing uncorrected data with matrix corrected; and
2. identifying the effects of matrix correction on laboratory mean concentrations.

The following observations were made with respect to the performance of the Cefas HPLC-MS/MS method under validation. It was assumed that (1) the process of matrix correction reduced quantitation bias and (2) the assigned concentrations were close to the true values:

- the quality control criteria (r^2 ≥0.98 and response drift ≤25%) were achieved for all toxin calibrations
- the EU-RL (2010) report stated there was no statistical difference between OA, PTX2, AZA1 and YTX concentrations obtained from the either calibrations prepared from commercially available, certified reference standards of these toxins or from their calibrations derived from the use of the pre-release, multi-toxin standard solution
- comparing Cefas laboratory, uncorrected OA concentrations to MMS corrected data (Table 35), matrix effects were negligible for this toxin in uncooked mussel and razor clam extracts. There was evidence of ~14% OA enhancement from the analysis of the cockle sample. After MMS correction, Cefas laboratory OA means were seen to be slightly higher than MMS corrected assigned means for the mussel and Razor clam samples. The opposite was found for the cockle sample where the laboratory mean was ~20% less than the assigned value
- uncorrected total DTX1 concentrations determined indirectly by OA calibrations were higher in the uncooked mussel sample compared to values obtain by direct (DTX1) quantitation. Respectively, apparent enhancement influences were 31 and 14% depending on the method of calibration. After applying MMS correction, these effects were reduced and laboratory and assigned mean data were similar

Table 35. Uncorrected and matrix corrected laboratory and assigned mean OA and DTX mean concentrations (determined after hydrolysis) reported from the EU-RL collaborative study.

Toxin	Shellfish species	Calibration	Uncorrected mean (µg/kg)		MMS corrected mean (µg/kg)		Mus-b corrected mean (µg/kg)	
			lab.	assigned	lab.	assigned	lab.	assigned
Total OA	Razor clam	OA	76.5	83.4	75.9	64.1	93.3	85.8
	Mussel (uncooked)		76.5	80.1	75.8	66.1	93.2	82.1
	Cockle		104	141	91.4	114	117	137
Total DTX1	Mussel	DTX1	275	388	241	251	345	301
		OA	330	422	251	249	340	312
Total DTX2	Razor clam	DTX2	59.7	65.1	56.1	53.3	72.7	64.4
		OA	57.3	68.2	48.9	51.1	70.1	69.8
	Cockle	DTX2	55.9	90.1	55.7	63.7	65.5	89.7
		OA	75.1	104	58.9	63.1	80.6	98.2

- For the razor clam sample, uncorrected and MMS corrected DTX2 concentrations obtained by direct quantitation differed by only 6%. Uncorrected values obtained by indirect, OA calibration were higher than MMS corrected and by 17%. For the cockle sample, there was no difference between uncorrected and MMS corrected DTX2 data when direct quantitation was applied. As with the Razor clam sample, by indirect quantitation, higher uncorrected quantities were found and indicated an

enhancement effect of ~28%. The application of direct quantitation and MMS correction thus appeared to reduce matrix influences on quantitation

- For all samples, the use of Mus-b matrix correction resulted in higher laboratory (and assigned) mean values levels of total OA, DTX1 and DTX2. It is questionable as to whether this approach to matrix correction is appropriate. Firstly, the extract is required to be diluted with methanol solvent and in the range of 1/6th for DTX1 and 1/50th for OA. The possibility of 'diluting-out' inherent matrix exists thus reducing its efficiency for the purpose of matrix correction. Secondly, the Mus-b extract does not contain DTX2. Matrix correction for this toxin in real sample extracts is thus achieved indirectly by using OA or DTX1 signal responses
- With reference to Table 36, PTX2 was only quantified in the wedge shell and striped Venus clam samples. For both samples and when comparing uncorrected laboratory mean concentrations with MMS corrected, approximately 20% signal enhancement was suspected. After MMS correction, laboratory PTX2 mean values were lowered. For the striped clam both laboratory and assigned mean values were equivalent after matrix correction. However and for the wedge shell clam sample, the laboratory mean was lower (by ~18%) than the assigned mean. This may have been due to differences in matrix effects between the two clam species

Table 36. Uncorrected and matrix corrected laboratory and assigned mean PTX2, AZA and YTX concentrations reported from the EU-RL collaborative study.

Toxin	Shellfish species	Calibration	Uncorrected mean (µg/kg)		MMS corrected mean (µg/kg)	
			lab.	assigned	lab.	assigned
PTX2	Wedge clam	PTX2	92.2	85.0	76.7	93.4
	Striped clam		102	88.3	83.5	78.9
AZA1	Razor clam	AZA1	51.6	50.0	47.2	50.6
	Mussel (uncooked)		385	348	352	339
	Cockle		40.2	41.4	40.4	43.8
	Wedge clam		86.4	81.4	79.0	88.4
	Striped clam		125	111	120	112
AZA2	Razor clam	AZA2	37.4	36.0	33.7	39.2
		AZA1	42.9	39.5	EU-RL data not provided	
	Wedge clam	AZA2	24.8	22.9	22.3	26.5
		AZA1	30.0	30.3	EU-RL data not provided	
	Mussel (uncooked)	AZA2	91.5	74.8	82.3	75.7
		AZA1	98.3	84.4	EU-RL data not provided	
	Cockle	AZA2	39.8	36.9	40.5	39.6
		AZA1	39.6	40.0	EU-RL data not provided	
AZA3	Mussel (uncooked)	AZA3	19.0	22.9	17.5	21.7
		AZA1	<LOQ	30.1	EU-RL data not provided	
YTX	Mussel (uncooked)	YTX	138	108	105	103

- Influences of matrix effects on the quantitation of AZA toxins in mussel, cockle and razor clam samples were observed to be generally weak and ≤10%. MMS corrected, laboratory means derived for AZA1, and AZA2 and AZA3 (by direct quantitation) were similar to assigned data. Unfortunately, laboratory and assigned MMS corrected concentrations obtained by indirect quantitation had not been provided in the EU-RL (2010) report to permit reporting here. However, it would appear MMS matrix correction had little impact on AZA1 concentration data, and AZA2 and AZA3 values determined by direct quantitation
- YTX was only measured in the uncooked mussel sample and on comparing uncorrected laboratory means with MMS corrected values, ~28% enhancement was

evident. Matrix correction after applying the MMS approach reduced this, and both laboratory and assigned means were similar.

Findings from Cefas' participation in both the RIKILT and EU-RL collaborative studies have highlighted the following:

- for the determination of DTX1 and DTX2 toxins in shellfish tissues representative of those species commonly tested in the UK, it is necessary that levels of these toxins are derived from direct quantitation rather than by indirect means using OA calibrations. This was reflected in the closeness of laboratory and assigned mean concentrations. In most instances, deploying the latter approach resulted in a positive bias of concentration data. The approach of direct quantitation for DTX1 and DTX2 was applied throughout the in-house validation exercises
- in general, there was evidence from the performance of the Cefas HPLC-MS/MS method that, on deploying a matrix matched standard prepared in a mussel extract to correct for matrix influences on DTX1, this appeared to result in a reduction of matrix influences. This was not only apparent for mussel samples but also from extracts of other shellfish species
- there was little difference between AZA2 concentrations determined by either direct or indirect quantitation and this suggests that AZA2 could be determined by both routes. However, a pronounced effect was apparent when AZA3 concentrations were derived indirectly from AZA1 calibrations. In this instance, values were considerably lower by ~1.5 times compared to values obtained from direct quantitation
- comparing the performance of the HPLC-MS/MS method to the quantitation of YTX, the method appeared to perform satisfactory during the EU-RL study. Matrix correction for a level of contamination at 10% of the regulatory limit was possible. However for higher levels of YTX that were required to be measured in samples from the RIKILT study, it would seem that matrix correction lacked a degree of effectiveness for this toxin.

The performance of the HPLC-MS/MS method in the QUASIMEME proficiency exercises and Cefas' *z*-scores derived from the BVL study show that method is capable of achieving satisfactory results. Cefas will continue to participate in future QUASIMEME rounds and will apply the new *Xevo TQ* instrument alongside the older *Quattro Micro* system to sample analysis. Assessing the outcomes from these exercises will strengthen our understanding of the method's performance and will aid in working towards improving the quality of our analytical data.

Valuable information on the application of matrix correction was gained from our participation in the RIKILT and EU-RL collaborative studies. It would appear matrix correction improves the quality of our data, especially for the measurement of OA, DTXs and AZA3. However, it will be necessary to verify these observations by undertaking further work using both instruments to examine the practicalities and effectiveness of applying this approach.

4.0. Summary of method performance characteristics.

- An in-house, HPLC-MS/MS method validation scheme for the measurement of marine lipophilic toxins (MLTs) in shellfish was undertaken. The aim was to establish method performance characteristics for the detection and quantitation of twelve regulated toxins in shellfish species commonly tested in UK MLT monitoring programmes. Species included: Common mussel; Common cockle; Pacific and Native oysters; King and Queen scallops; Hard and Razor clams. With the advent of new EU regulation (Anon, 2011), the mouse bioassay used to monitor these toxins is to be replaced by LC-MS/MS. Method validation was to provide an suitable analytical tool to meet the new regulation and the demands from monitoring programmes
- An LC-MS/MS method (Gerssen *et al*., 2009) using an alkaline gradient was selected and adapted to an Agilent *1100* high performance liquid chromatograph (HPLC) and Waters *Quattro Micro* tandem mass spectrometer. After method optimisation, refinement and using multiple reaction monitoring of two transition ions per analyte, simultaneous detection of the regulated MLTs was achieved within 22.5 min
- To isolate MLTs from shellfish tissues, the EU Reference Laboratory for Marine Biotoxins (EU-RL, 2006) extraction procedure was applied. A pre-validation exercise showed this to be suitable for the quantitative recovery of free OA/DTXs, PTX2, AZA1/2/3, YTX/45 OH YTX and the non-regulated toxin, SPX1
- The HPLC-MS/MS method demonstrated satisfactory selectivity for the detection of all regulated toxins and no visible interfering chromatographic peaks were evident at or close to expected retention times of the target analytes
- Acceptable linearity of instrumental response was demonstrated for the majority of toxins (OA/DTXs, PTX2/11, AZA1/2/3 and non-regulated SPX1 and GYM) prepared as solvent-based and shellfish matrix matched calibration standards. Correlation coefficients were all ≥0.98, visual examinations of calibration plots and assessments from statistical, lack-of-fit F-tests supported this performance. Where lack-of-fit tests failed, indicating non-linearity, these were due to slight bias of a single calibration point. With the exception of YTX, where a linear range equivalent of 10-150% of the regulatory limit (RL) was found, linear ranges for other toxins were <10 to 200% RL
- The HPLC-MS/MS method appeared to be prone to a complex array of matrix influences. It was difficult to identify patterns of matrix effects on a single toxin or groups of toxins that were common to a particular shellfish species or groups of species. Matrix influences ranged from negligible effects, strong enhancement effects on YTX responses, and strong suppressions exerted on PTX2
- Variations of shellfish matrix influences on regulated lipophilic toxins were described from the analysis of spiked mussel and Pacific oyster extracts. These were prepared from samples acquired at different times and from different shellfish producing areas. Between-sample differences in average responses for OA, DTX1, DTX2, PTX2 and AZA1 existed. However, YTX was found to be enhanced during the HPLC-MS/MS analysis of both mussels and oysters extracts. Diluting-out matrix interferences produced a complexity of results. The approach appeared to reduce effects on some toxins, whilst for other analytes, no impact was found. In some cases, opposite matrix influences were induced after dilution. As a method for moderating or controlling matrix interferences, extract dilution did not appear to be a feasible option. Applying a solid phase extraction (SPE) clean up method to mussel and oyster extracts was only partially effective in reducing matrix influences on

OA/DTXs, PTX2 and AZA1. However, reductions were not reproduced for these analytes in all samples. Clean up eliminated enhancement influences on OA/DTXs in crude oyster extracts, although during analysis of cleaned extracts, suppression effects were exerted on the toxins. Crude extract suppression effects on PTX2 were exaggerated after SPE clean up. However, SPE clean up did appear to be a possible solution in overcoming co-extracted mussel and oyster matrix influences exerted on YTX. However, the variable performance of the clean up method makes this option less efficient in reducing matrix influences exerted on other toxins

- Experimentally determined method LODs for individual OA/DTXs in eight shellfish species were 7 µg[DTX2]/kg (Pacific oyster) to 16 µg[DTX2]/kg (Razor clam). LOQs ranged from 23 µg[DTX1]/kg (mussel) to 58 µg[OA]/kg (Native oyster). The HPLC-MS/MS method was least sensitive to quantifying these analytes compared to LOQs achieved for other toxins. On applying TEFs, OA/DTX1 LOQs represented 52 to 76% RL of 160 µg[OA eq.]/kg. Respectively, LOQs for PTX2 and AZA1 were ≤10 and ≤5 µg/kg. For YTX, LOQs were <11% RL of 1 mg/kg and ranged from 28 µg/kg (P. oyster) to 105 µg/kg (mussel)
- Method recoveries were determined from extracted spiked shellfish tissues. OA was spiked at 38% RL, DTX1/2, PTX2, and AZA1 at 63% RL and YTX at 25% RL. Low level spiking and close to method LOQ values, was also undertaken. A majority of recoveries met the 2002/657EC criteria of -20% to 10% for concentrations ≥10 µg/kg. An average recovery of 93% was calculated for OA/DTXs, PTX2, AZA1 and YTX toxins extracted from all tissues of interest. Toxin recoveries from tissues spiked with 'high' levels ranged from 74% (YTX/mussel) to 127% (YTX/Razor clam). Co-extracted matrix suppression influences may have been responsible for slightly lower recoveries exhibited by PTX2 (range 82-97%) and AZA1 (80-92%). Range of recoveries from tissues fortified close to method LOQs was 71% (DTX1/mussel) to 671% (YTX/R. clam). Severe matrix enhancement effects would have most likely biased recoveries of YTX particularly from Queen scallop and clam tissues
- Method repeatability (within-batch precision) experiments involved one laboratory analyst extracting and analysing replicated spiked shellfish tissues in the range of 60-250 µg/kg and at levels close to method LOQ values. For tissues spiked at higher concentrations, RSD_rs were 2-17% for the determination of OA/DTXs, PTX2, AZA1, YTX and non-regulated SPX1 and GYM. Average percentage RSD_rs were considered acceptable; for OA/DTXs these were 7-9%, for PTX2 and AZA1 <5% and for YTX ~10%. Higher values were seen for YTX determinations from oysters, Queen scallop and R. clam shellfish. RSD_rs associated with the determination of MLTs at concentrations close to LOQs were higher for a majority of toxin/shellfish combinations. Within-laboratory method reproducibility studies were conducted over three months and involved three analysts performing spiking (at higher concentrations), extraction and analysis. Most RSD_Rs were acceptable and <11%, however YTX determinations produced higher RSD_Rs (range 9-21%; average 15%)
- A draft standard operating procedure (SOP) outlining the extraction procedure and method performance criteria based on the method characteristics is presented in Appendix 42
- To assess the stability of the entire (extraction *plus* analytical) method, ruggedness studies were undertaken and deliberate changes to method parameters were systematically introduced. Outcomes from statistical *t*-tests infer that none of the parameter changes had significant effects on the stability of the method. In comparison to method repeatability precision, a lower variability was associated with the determination of OA/DTXs and YTX following ruggedness experiments.

The reverse was apparent for PTX2 and AZA1 measurements. However and on the assumption the parameters do not interact, the method was consider rugged for determination of OA/DTXs, PTX2, AZA1 and YTX in shellfish tissues of interest

- Standardised and expanded measurement uncertainties for the measurement of OA/DTXs, PTX2/11, AZA1/2/3, YTX/45 OH YTX, and SPX1 and GYM were calculated from results of validation exercises carried out during this work. Combined standardised uncertainties ranged from 0.05 to 0.40 and mean values for each shellfish species were from 0.10 to 0.26. Expanded uncertainties (k=2) ranged from 0.09 (AZA1/King scallop) to 0.80 (YTX/mussel). It is important to note that these data are preliminary. Combined uncertainties are likely to change over time as more method performance data is obtained through routine implementation of the procedure and associated analytical quality control
- On transferring and converting the HPLC-MS/MS method to a newly acquired instrument deploying Ultra Performance LC (UPLC™) and 'fast' MS data acquisition, sample analysis time was reduced by 65%. This has a positive effect on sample throughput. Thus, the UPLC-MS/MS method is amenable to processing a high volume of samples and meeting demands of national monitoring programmes
- Visual assessments of calibration plots from solvent and matrix matched standards, examination of residuals of regressions and correlation coefficients being ≥0.99 all indicated an acceptable linear relationship between concentration and detector response. The working linear range was equivalent to 4-400% RLs for OA, DTX1/2, PTX2 and AZA1 toxins. For YTX, linear range extended to 4 times the RL and this was an improvement over the performance of the conventional HPLC-MS/MS method which is limited to 150% RL
- When applied to the analysis of OA/DTXs, PTXs and AZA1, the UPLC-MS/MS method appeared to be more susceptible to suppression influences derived from mussel tissue than from other shellfish matrices. Where YTX was found to be enhanced during HPLC-MS/MS analysis using the *Quattro Micro* mass spectrometer, this toxin was seen to be suppressed during UPLC-MS/MS analysis
- For all shellfish matrices, instrumental LOQs for OA/DTXs were estimated to be <10 µg/kg. On a TEF basis, summed LOQs were equivalent to 12% of 160 µg[OA eq.]/kg. Thus, sensitivity improvements were gained from the application of UPLC-MS/MS to this group of toxins. LOQs for PTX2 and PTX11 were similar (<3 µg/kg) and LOQs for YTX indicated that lower concentrations can be achieved by UPLC-MS/MS. UPLC- and HPLC-MS/MS methods were similar in sensitivity performance for AZA1
- Satisfactory statistical comparability was shown between UPLC- and HPLC-MS/MS for the quantitation of most regulated toxins [free OA/DTXs, total OA/DTX1 (after hydrolysis), PTX1/2 and AZA1/2/3] from naturally contaminated mussel, cockle and Pacific oyster tissues. There was some evidence to suggest that for samples contaminated with low (<20 µg/kg) concentrations, higher values were returned by the HPLC-MS/MS method
- Throughout the in-house validation period, the HPLC-MS/MS method was applied to test samples provided in 'QUASIMEME' proficiency exercises. Derived *z*-scores showed the method performs satisfactorily for the majority of measurements of free and total OA/DTXs and AZA1/2/3 in a range of shellfish matrices
- The HPLC-MS/MS method was also applied to test samples from three interlaboratory studies, organised by BVL (Germany), RIKILT (The Netherlands) and the EU-RL (Spain). In the BVL study involving the determination of OA/DTXs,

PTX2, AZAs and YTX toxins in cooked mussel tissues, oyster and clam extracts, *z*-scores of ≤|2| indicated the method performed satisfactorily and acceptable recoveries (94-106%) were achieved from spiked extracts

- In the RIKILT study, all participants were required to apply the alkaline LC gradient as adopted for the in-house validation scheme reported here. The analytical method met quality control criteria (r^2≥0.98; response drift ≤25%) for most toxin calibrations with the exception of total OA and DTX2 calibrations. Toxin quantities in mussel, oyster and cockle tissues were determined from a mussel matrix matched standard. Cefas laboratory mean concentrations of free OA were consistently higher than assigned mean values by ~1.2. By direct quantitation using DTX1 and DTX2 calibrations, free and total DTX1 and DTX2 levels were similar to assigned means. However by indirect (OA) quantitation, free DTX1 and DTX2, and total DTX1 concentrations were higher than assigned means by respective factors of ~1.5, 1.2 and 1.6. Recoveries of PTX2 from spiked tissues were ≥90%, and laboratory and assigned means were similar. Little difference was found between AZA2 laboratory and assigned means when this toxin was determined directly or indirectly using AZA2 and AZA1. However, quantitation of AZA3 concentrations by direct quantitation resulted in laboratory mean values being higher (~1.5) than assigned values. For two mussels and the cockle sample, YTX means were similar although for other mussel samples, laboratory means were higher. It is possible that the application of the matrix matched standard approach for the measurement of YTX was not as efficient as it was shown for DTXs
- During the EU-RL interlaboratory study, participants followed the EU-RL extraction procedure but were permitted to apply different LC-MS/MS methods. The Cefas HPLC-MS/MS method successfully achieved the quality control criteria (r^2≥0.98; response drift ≤25%) for all toxin (OA, DTX1/2, PTX2, AZA1/2/3 and YTX) calibrations. There was some evidence to show that mussel matched matrix correction of DTX1 and DTX2 concentrations in mussel and Razor clam samples and their direct quantitation resulted in a reduction of matrix influences exerted on these toxins. After matrix correction and direct quantitation, laboratory and assigned mean values were similar for the determination of AZAs in mussel, cockle and clam tissues. Matrix correction for YTX quantified from mussel samples also reduced an apparent enhancement effect on this analyte, and mean values were equivalent
- From Cefas' participation in these interlaboratory studies, valuable information was gained on the performance of the LC-MS/MS method. It was found that mussel matrix correction showed the potential to improve the quality of our toxin data derived from the analysis of a range of shellfish species including mussel, cockle, oyster and clams.

5.0. Conclusions and recommendations.

Cefas has successfully adapted, refined and validated an LC-MS/MS method permitting the simultaneous determination of regulated marine lipophilic toxins in those shellfish typically tested in the UK statutory monitoring programmes. Single laboratory method validation was performed in compliance with international guidance (IUPAC guidelines; Thompson *et al.* 2002)] as required in Regulation (EC) 882/2004 (Anon. 2004). Within the toxin/shellfish matrix combinations studied: the LC-MS/MS method demonstrated good specificity with a linear response over several orders of magnitude. The method was sufficiently sensitive with quantitation limits low enough to enable reliable determination of compliance with regulatory limits; recovery and within-laboratory precision were satisfactory as was ruggedness. The results obtained from interlaboratory and proficiency test studies demonstrated the applicability of the method to the analysis of lipophilic toxins present at a range of concentrations in tissues from different shellfish species. Suitable evidence of the reliability and reproducibility of the method from these studies was also shown.

The multi-toxin approach that combines a convenient and effective extraction procedure with the advantages of the specificity of LC-MS/MS analysis allows for efficient monitoring of the range of toxins commonly seen in shellfish produced in UK waters. Practical trials on field samples showed the method to be fit-for-purpose, amenable to automation and applicable for routine analysis. The deployment of rapid UPLC-MS/MS further shortens the analytical time, improves method sensitivity and would be recommended for high sample throughput.

The disadvantages of the current biological reference method (mouse bioassay) are fully documented in reports produced by the European Food Safety Authority. Our findings demonstrate that the LC-MS/MS method can be regarded as a suitable alternative with advantages in respect to sensitivity, toxin identification, quantitation, and confirmation. To the best of our knowledge, the validated LC-MS/MS method meets the requirements of new European legislation and the necessity to replace the existing reference method.

In summary and from the technical perspective, the LC-MS/MS method is practical and fit-for-purposes and we recommend that it should be implemented in the UK lipophilic toxin monitoring programmes for the following species: common mussels (*Mytilus edulis*); common cockles (*Cerastoderma edule*); Pacific oyster (*Crassostrea gigas*); native oyster (*Ostrea edulis*); King scallops (*Pecten maximus*); Queen scallops (*Aequipecten opercularis*); hard clams (*Mercenaria mercenaria*); and razor clams (*Enis* spp.). Information relating to method properties is not currently available to evaluate performance against minor species (surf clams, otter shell clams, carpet clams, manila clams and processed scallops), however these currently constitute <3% of samples tested in Great Britain.

For a laboratory to implement the LC-MS/MS method into a routine monitoring programme, the Competent Authority needs to determine the approach to various practical issues. These include: result turnaround requirements; sample testing capacities; quality control criteria; approach for application of toxicity equivalent factors, recovery correction and result uncertainty; reporting format; contingency requirements; and response to novel and emerging toxins.

Following implementation, Cefas recommends additional work to further refine and optimise the application of the method in respect to: the need for and effectiveness of the application of matrix correction using matrix matched standards (arising from findings from

our participation in interlaboratory studies); the incorporation of new lipophilic toxin reference standards (*e.g.,* AZA2, AZA3) as they become commercially available and an assessment of LC-MS/MS method performance relating to these; and validation performance checks for minor shellfish species to allow the method to be extended and applied to all official control samples acquired under the national shellfish monitoring programmes.

6.0. References

Aasen, J.A.B., Samdal, I.A., Miles, C.O., Dahl, E., Briggs, L.R., Aune, T. (2005a). Yessotoxins in Norwegian blue mussels (*Mytilus edulis*): uptake from *Protoceratium reticulatum*, metabolism and depuration. *Toxicon*, **45**, 265.

Aasen, J.A.B., MacKinnon, S.L., LeBlanc, P., Walter, J.A., Hovgaard, P., Aune, T., Quilliam, M.A.. (2005b). Detection and identification of spirolides in Norwegian shellfish and plankton. *Chem. Res. Toxicol*., **18**, 509.

Alvarez, G., Uribe, E., Avalos, P., Marino, C., Blanco, J. (2010). First identification of azaspiracid and spirolides in *Mesodesma donacium* and *Mulinia edulis* from Northern Chile. *Toxicon*, **55**, 638.

AMC (1994). Is my calibration linear? *Analyst*, **119**, p.2363

Amzil, Z., Sibat, M., Royer, F., Savar, V. (2008). First report on azaspiracid and yessotoxin groups detection in French shellfish. *Toxicon*, **52**, 39.

Armitage, P., Berry, G. (1994). *Statistical Methods in Medical Research* (3rd edition). Blackwell.

Anon (1986). Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of Member States regarding the protection of animals for experimental and other scientific purposes. *Official Journal of the European Union,* **L358.** Articles 7 and 23.

Anon (2000). Quantifying uncertainty in analytical measurement. Eurachem/Citac guide, 2nd edition. Eds: S.L.R. Ellison, M. Rosslein, A. Williams.

Anon. (2002). The Commission of the European Communities. (2002). Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal of the European Union,* **L221**, 8-36.

Anon. (2004a). Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. *Official Journal of the European Union,* **L139**. Section VII, Chapter V. 60-61.

Anon. (2004b). Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. *Official Journal of the European Union,* **L165.** Annex III, p. 120.

Anon. (2005). Regulation (EC) No 2074/2005 of the European Parliament and of the Council of 5 December 2005 laying down implementing measures of certain products under Regulation (EC) No 835/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/200 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. *Official Journal of the European Union* **L338.** Annex III, Chapter III. pp 40-41

Anon. (2011). Commission Regulation (EU) 15/2011 of 10 January 2011 amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalves. . *Official Journal of the European Union*, **L6.** 3-6.

Aune, T., Sorby, R., Yasumoto, T., Ramstad, H., Landsverk, T. (2002). Comparison of oral and intraperitoneal toxicity of yessotoxin towards mice, *Toxicon*, 4**0**, 77.

Donnell, G.E. and Hibbert D.B. (2005). Treatment of bias in estimating measurement uncertainty. *The Analyst*. **130**, 721-729.

Draisci, R., Ferretti, E., Palleschi, L., Marchiafava, C., Polleti, R., Milandri, A., Ceredi, A., Pompei, M. (1999). High levels of yessotoxin in mussels and presence of yessotoxin and homoyessotoxin in dinoflagellates of the Adriatic Sea. *Toxicon,* **37**, 1187.

Elgarch, A., Vale, P., Rifai, S., Fassouane, A. (2008). Detection of diarrheic shellfish poisoning and azaspiracid toxins in Maroccan mussels: comparison of the LC-MS method with the commercial immunoassay kit. *Mar Drugs*, **6**, 587.

Espina, B., Rubiolo, J.A. (2008). Marine toxins and the cytoskeleton: pectenotoxins, unusual macrolides that disrupt actin. *FEBS J*., **275**, 6082.

European Food Safety Authority. (2008a). Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on marine biotoxins in shellfish – okadaic acid and analogues. *The EFSA Journal*, **589**, 1-62.

European Food Safety Authority. (2008b). Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on Marine biotoxins in shellfish – yessotoxin group. *The EFSA Journal*, **907**: 1-62.

European Food Safety Authority. (2008c). Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on Marine biotoxins in shellfish –azaspiracid group. *The EFSA Journal*, **723**, 1-52

European Food Safety Authority. (2009). Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on marine biotoxins in shellfish - pectenotoxin group. *The EFSA Journal*, **1109**, 1-47.

European Reference Laboratory for marine biotoxins (EU-RL; 2006). Standard operating procedure for the processing and extraction of shellfish for the subsequent detection of diarrhetic shellfish poisons (okadaic acid and dinophysistoxins), and azaspiracids and pectenotoxins in bivalve molluscs. August 2006. pp. 30.

European Reference Laboratory for marine biotoxins (EU-RL; 2009). EU harmonised standard operating procedure for detection of lipophilic toxins by mouse bioassay, version 5 June 2009. http//www.aesan.msps.es/en/CRLMB/web/home.shtml (15-07-2009).

European Reference Laboratory for marine biotoxins (EU-RL; 2010). Interlaboratory validation study of the 'EU harmonised standard operating procedure for the determination of lipophilic marine biotoxins in bivalve molluscs by LC-MS/MS. Draft report (November 2010). pp. 20.

Food and Agriculture Organization. (2005). Joint FAO/IOC/WHO ad hoc Expert Consultation on Biotoxins in Bivalve Molluscs, Background Document, Oslo, Norway, September 26-30, 2004.

Fux, E., McMillan, D., Bire, R., Hess, P. (2007). Development of an ultra-performance liquid chromatography-mass spectrometry method for the detection of lipophilic marine toxins. *J Chromatogr A.,* **1157**, 273.

Fux, E., Rode, D., Bire, R., Hess, P. (2008). Approaches to the evaluation of matrix effects in liquid chromatography-mass spectrometry (LC-MS) analysis of three regulated lipophilic toxin groups in mussel matrix (*Mytilus edulis*). *Food Additives and Contaminants*, **25**, 1024-1032.

Garcia, C., Truan, D., Lagos, M., Santelices, J.P., Diaz, J.C., Lagos, N. (2005). Metabolic transformation of dinophysistoxin-3 into dinophysistoxin-1 causes human intoxication by consumption of O-acyl-derivatives dinophysistoxins contaminated shellfish. *J. Toxicol. Sci*., **30**, 287.

Gerssen, A., Mulder, P. and de Boer, J. (2007). A novel LC method for the separation of marine lipophili biotoxins. *Third International Symposium on Recent Advances in Food Analysis*. 7 to 9 November 2007, Prague, Czech Republic. p. 249.

Gerssen, A., Mulder, P.P.J., McElhinney, M.A. and de Boer, J. (2009). Liquid chromatography–tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. *J. Chromatogr. A*, **1216**, 1421–1430

Gerssen, A., van Olst, E.H.W., Mulder, P.P.J., de Boer, J. (2010). In-house validation of a liquid chromatography tandem mass spectrometry method for the analysis of lipophilic marine toxins in shellfish using matrix matched calibration. *Anal. Bioanal. Chem*. DOI 10.1007/s00216-010-3886-2

Heil, D.C. (2009). Karenia brevis monitoring, management, and mitigation for Florida molluscan shellfish harvesting areas. *Harmful Algae*, **8**, 608.

Honkanen, R.E., Codispoti, B.A., Tse, K., Boynton, A.L., Honkanan, R.E. (1994). Characterization of natural toxins with inhibitory activity against serine/threonine protein phosphatases. *Toxicon,* **32**, 339.

Hoagland, P., Scatasta, S., (2006). The economic effect of harmful algal blooms. In: Ecology on Harmful Algae, (Eds): E. Graneli, T. Turner T. Springer. Berlin. Vol. 189, pp 391.

International Conference on Harmonisation Tripartite Guideline. (2005). ICH Topic Q2, Validation of Analytical Procedures: Text and Methodology, Geneva, Switzerland. Accessible at http://www.ich.org/cache/compo/276-254-l.html

International Organisation for Standardisation (1995). Guide to the expression of uncertainty in measurement. ISBN 92-67-10188-9. Geneva, Switzerland.

Ishida, H., Nozawa, A., Nukaya, H., Tsuji, K. (2004). Comparative concentrations of brevetoxins PbTx-2, PbTx-3, BTX-B1 and BTX-B5 in cockle, *Austrovenus stutchburyi*, greenshell mussel, *Perna canaliculus*, and Pacific Oyster, *Crassostrea gigas*, involved neurotoxic shellfish poisoning in New Zeland. *Toxicon,* **43**, 779.

James, K.J., Lehane, M., Maroney, C., Fernandez-Puente, P., Satake, M., Yasumoto, T., Furey, A. (2002). Azaspiracid shellfish poisoning: unusual toxin dynamics in shellfish and the increased risk of acute human intoxications. *Food Addit. Contam.* **19**, 555.

James, K.J., Saez, M.J.F., Furey, A., Lehane, M. (2004). Azaspiracid poisoning, the food-borne illness associated with shellfish consumption. *Food Addit. Contam*., **21**, 879.

Klontz, K.C., Abraham, A., Plakas, S.M., Dickey, R.W. (2009). Mussel-associated azaspiracid intoxication in the United States. *Ann Intern Med*., **150**, 361.

Lacaze, J.-P.C.L., Stobbo, L.A., Turrell, E.A., Quilliam, M.A. (2007). Solid phase extraction and liquid chromatography mass spectrometry for the determination of free fatty acids in shellfish. *J. Chromatogr. A*, 1145, 51-57.

Landsberg, J.H., Flewelling, L.J., Naar, J. (2009). *Karenia brevis* red tides, brevetoxins in the food web, and impacts on natural resources: Decadal advancements. *Harmful Algae*, **8**, 598.

Lira, I.H., Wöger W. (1998). Evaluation of the uncertainty associated with a measurement result not corrected for systematic effects. *Meas. Sci. Technol*. **9**: 1010-1011.

Lopez-Rivera, A., O'Callaghan, K., Moriarty, M., O'Driscoll, D., Hamilton, B., Lehane, M., James, K.J., Furey A. (2009). First evidence of azaspiracids (AZAs): A family of lipophilic polyether marine toxins in scallops (*Argopecten purpuratus*) and mussels (*Mytilus chilensis*) collected in two regions of Chile. *Toxicon*, **55**, 692.

Magnusson, B., Ellison, S.L.R. (2008). Treatment of uncorrected measurement bias in uncertainty estimation for chemical measurements. *Anal. Bioanal. Chem*., **390**: 201-213

McCarron, P., Giddings, S., Quilliam, M.A. (2009). CRM-FDMT1, a candidate CRM for the analysis of multiple groups of shellfish toxins: characterisation of short- and long-term stability. CRMP Technical Report FDMT1-20090517-01. May 17 2009.

McNabb, P., Selwood, A.L., Holland, P.T. (2005). Multiresidue method for determination of algal toxins in shellfish: Single-laboratory validation and inter-laboratory study. *J. AOAC Int*., **88**, 761.

Miles, C.O., Wilkins, A.L., Munday, R., Dines, M.H., Hawkes, A.D., Briggs, L.R., Sandvik, M,, Jensen, D.J., Cooney, J.M., Holland, P.T., Quilliam, M.A., Mackenzie, A.L., Beuzenberg, V., Towers, N.R.. (2004). Isolation of pectenotoxin-2 from *Dinophysis acuta* and its conversion to pectenotoxin-2 seco acid, and preliminary assessment of their acute toxicities. *Toxicon*, **43**, 1.

Miles, C.O., Samdal, I.A., Aasen, J.A.B., Jensen, D.J., Quilliam, M.A., Petersen, D., Briggs, L.M., Wilkins, A.L., Rise, F., Cooney, J.M., Mackenzie, A.L.. (2005). Evidence for numerous analogs of yessotoxin in *Protoceratium reticulatum*. *Harmful Algae*, **4**, 1075.

Miller, G. (2009). Wildlife biology – confused pelicans may have lingered too long up north. *Science*, **323**, 449.

Mountfort, D.O., Suzuki, T. Truman, P. (2001). Protein phosphatase inhibition assay adapted for determination of total DSP in contaminated mussels. *Toxicon* 39, 383-390.

Plackett, R.L., Burman, J.P. (1946). *Biometrika*, 33, 305-325.

Quilliam, M.A., Hess, P., Dell'Aversano, C. (2001). Recent developments in the analysis of phycotoxins by Liquid Chromatography-Mass Spectrometry. In *Mycotoxins and Phycotoxins in Perspective at the Turn of the Century*. In: W.J. deKoe, R.A. Samson, H.P. van Egmond, J. Gilbert and M.Sabino (Eds), Netherlands, Proceedings of the Xth International IUPAC Symposium on Mycotoxins and Phycotoxins, Sao Paulo, Brazil, May 22-25 2000. pp. 383-391.

Rehmann, N., Hess, P., Quilliam, M.A. (2008). Discovery of new analogs of the marine biotoxin azaspiracid in blue mussels (*Mytilus edulis*) by ultra-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom*., **22**, 549.

Richard, D.J.A., Arsenault, E., Cembella, A.D., Quilliam, M.A. (2000). Investigations into the toxicology and pharmacology of spirolides, a novel group of shellfish toxins. In: Proceedings of the 9th conference on Harmful algal blooms, G.M. Hallegraeff, S.I. Blackburn, C.J. Bolch, R.J. Lewis, (Eds.) Hobart, Australia, 2000; pp 383.

Seki, T., Satake, M., Mackenzie, A.L., Kaspar, H.F., Yasumoto, T. (1995). Gymnodimine, a new marine toxin of unprecedented structure isolated from New-Zealand oysters and the dinoflagellate, *Gymnodinium* Sp. *Tetrahedron Lett*., **36**, 7093.

Scoging, A., Gahl, M. (1998). Diarrhetic shellfish poisoning in the UK. *The Lancet*, **352**, 117.

Stobo, L.A., Lacaze, J.P.C.L., Scott, A.C., Gallacher, S., Smith, E.A., Quilliam, M.A. (2005). Liquid chromatography with mass spectrometry – detection of lipophilic shellfish toxins. *J. AOAC Int*., **88**, 1371-1382.

These, A., Scholz, J., Angelika Preiss-Weigert, A. (2009). Sensitive method for the determination of lipophilic marine biotoxins in extracts of mussels and processed shellfish by high-performance liquid chromatography–tandem mass spectrometry based on enrichment by solid-phase extraction. *J. Chromatogr. A*., **1216**, 4529-4538.

These, A., Klemm, C., Nausch, I., Uhlig, S. (2010). Results of a European interlaboratory method validation study for the quantitative determination of lipophilic marine biotoxins in raw and cooked shellfish basedon high-performance liquid chromatography–tandem mass spectrometry. Part I: collaborative study. *Anal. Bioanal. Chem*. DOI 10.1007/s00216-010-4383-3

Thompson, .M., Ellison, S.L., Wood, R. (2002). Harmonised guidelines for single validation of methods of analysis (International Union of Pure and Applied Chemistry technical report). *Pure Appl. Chem*., **5**, 835-855.

Torgersen, T., Bremnes, N.B., Rundberget, T., Aune, T. (2008). Structural confirmation and occurrence of azaspiracids in Scandinavian brown crabs (*Cancer pagurus*). *Toxicon*, **51**, 93.

Uhlig, S., Kirsten, S. Antoni, S. (2010). Summary of results of the collaborative method validation study "Marine Lipophilic Biotoxins" in the framework of the § 64 LFGB (German Food and Feed Act), working group Phycotoxins. Final draft report. Reported by quodata (www.quodata.de). pp 143.

Vale, P., Sampayo, M.A.D. (2002). Pectenotoxin-2 seco acid, 7-epi-pectenotoxin-2 seco acid and pectenotoxin-2 in shellfish and plankton from Portugal. *Toxicon,* **40**, 979.

Vale, P., Bire, R., Hess, P. (2008a). Confirmation by LC-MS/MS of azaspiracids in shellfish from the Portuguese north-western coast. *Toxicon*, **51**, 1449.

Vale, P., Botelho, M.J., Rodrigues, S.M., Gomes, S.S., Sampayo, M.A.D. (2008b). Two decades of marine biotoxin monitoring in bivalves from Portugal (1986-2006): A review of exposure assessment. *Harmful Algae*, **7**, 11.

Vale, P., Gomes, S.S., Lameiras, J., Rodrigues, S.M., Botelho, M.J., Laycock, M.V. (2009). Assessment of a new lateral flow immunochromatographic (LFIC) assay for the okadaic acid group of toxins using naturally contaminated bivalve shellfish from the Portuguese coast. *Food Addit. Contam*., A, **26**, 214.

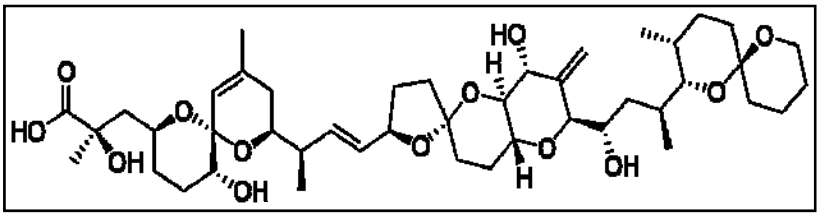
van den Top, H., Gerssen, A., van Egmond. (2010). International validation study of the method: Quantitative determination of marine lipophilic toxins in shellfish using LC-MS/MS. Draft report (November 2010). Institute of Food Safety (RIKILT), The Netherlands. p.p. 53.

Villar-González, A., Rodríguez-Velasco, M.L., Ben-Gigirey, B., Yasumoto, T., Botana, L.M., (2008). Assessment of the hydrolysis process for the determination of okadaic acid-group toxin ester: presence of okadaic acid 7-*O*-acyl-ester derivatives in Spanish shellfish. *Toxicon* **51**, 765–773.

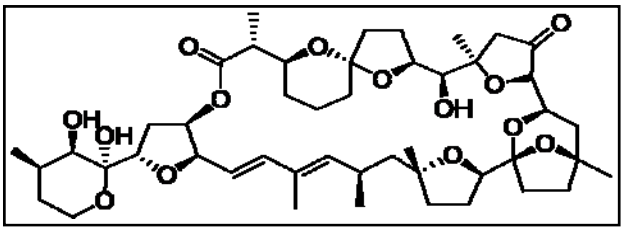
Watkins, S.M., Reich, A., Fleming, L.E., Hammond, R. (2008). Neurotoxic shellfish poisoning. *Mar. Drugs*, **6**, 431.

Appendices.

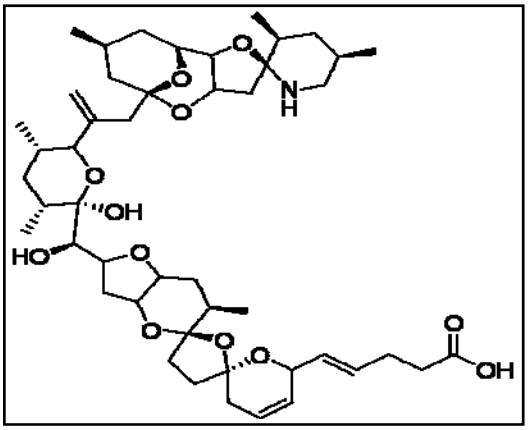
Appendix 1. Chemical structures of key regulated and non-regulated marine lipophilic toxins.



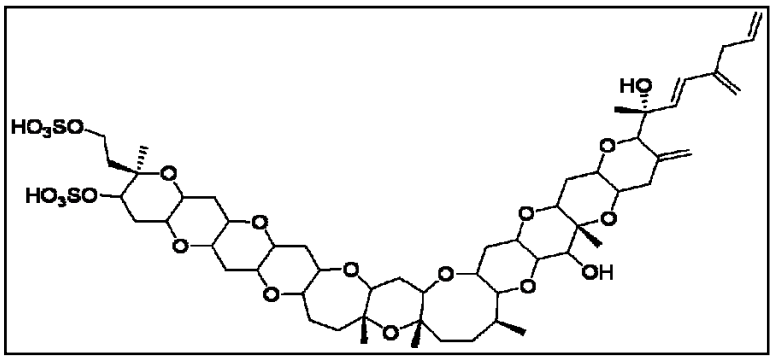
Okadaic acid (OA)



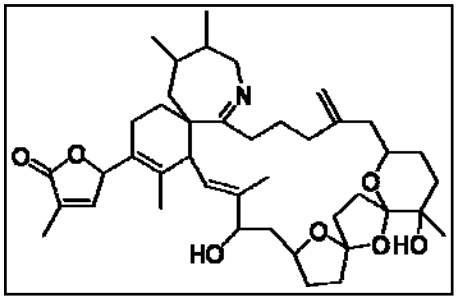
Pectenotoxin 2 (PTX2).



Azaspiracid 1 (AZA1)



Yessotoxin (YTX)



13-desmethyl spirolide C (SPX1)

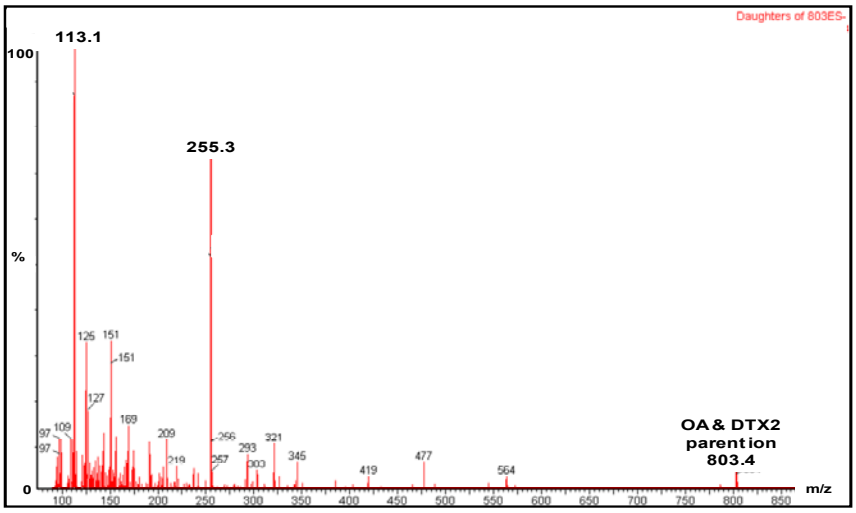
Appendix 2. Optimised *Quattro Micro* (Waters Ltd., UK) mass spectrometric source cone voltages and collision energies (CE) for the production of precursor and transition (fragment) ions for regulated and non-regulated (highlighted) marine lipophilic toxins.

Toxin	MW (amu)	Ionisation mode	Pseudo-molecular ion	Precursor ion m/z	Transition ions m/z	Cone (V)	CE (eV)
OA	804.5	-ve	$[M-H]^-$	803.4	113.1	77	61
					255.3	77	61
DTX1	818.5	-ve	$[M-H]^-$	817.4	113.1	77	64
					255.3	77	62
DTX2	804.5	-ve	$[M-H]^-$	803.4	113.1	77	61
					255.3	77	61
PTX1	876.5	+ve	$[M+NH_4]^+$	892.6	213.3	35	35
					821.5	35	33
PTX11*	876.5	+ve	$[M+NH_4]^+$	892.6	213.3	35	35
					821.5	35	33
PTX2	858.5	+ve	$[M+NH_4]^+$	876.4	213.2	35	34
					823.4	35	31
PTX2*sa* + 7-*epi* PTX2*sa*	876.5	-ve	$[M-H]^-$	875.5	136.9	65	57
YTX	1142.5	-ve	$[M-2H]^{2-}$	570.4	396.2	41	37
					467.2	41	37
***homo* YTX**	1156.5	-ve	$[M-2H]^{2-}$	577.4	403.4	41	37
					474.4	41	37
45 OH YTX	1158.5	-ve	$[M-2H]^{2-}$	578.4	396.2	41	37
					467.2	41	37
45 OH *homo* YTX	1172.5	-ve	$[M-2H]^{2-}$	585.4	403.4	41	37
					474.4	41	37
AZA1	841.5	+ve	$[M+H]^+$	842.4	654.3	42	52
					362.3	42	52
AZA2	855.5	+ve	$[M+H]^+$	856.4	362.5	47	57
					654.5	47	55
AZA3	827.5	+ve	$[M+H]^+$	828.3	362.5	41	57
					658.5	41	55
GYM	507.5	+ve	$[M+H]^+$	508.4	162.0	33	40
					392.4	33	36
SPX1	691.5	+ve	$[M+H]^+$	692.5	177.1	49	45
					444.3	49	41

* isomer of PTX1
MW = molecular weight
amu = (mono-isotopic) atomic mass unit
eV = electron volts
V = volts
MS (data acquisition) file
Dwells 80 to 150 ms
Inter Channel Delay = 0.05 s
Inter Scan Time = 0.1 s
Repeats = 1

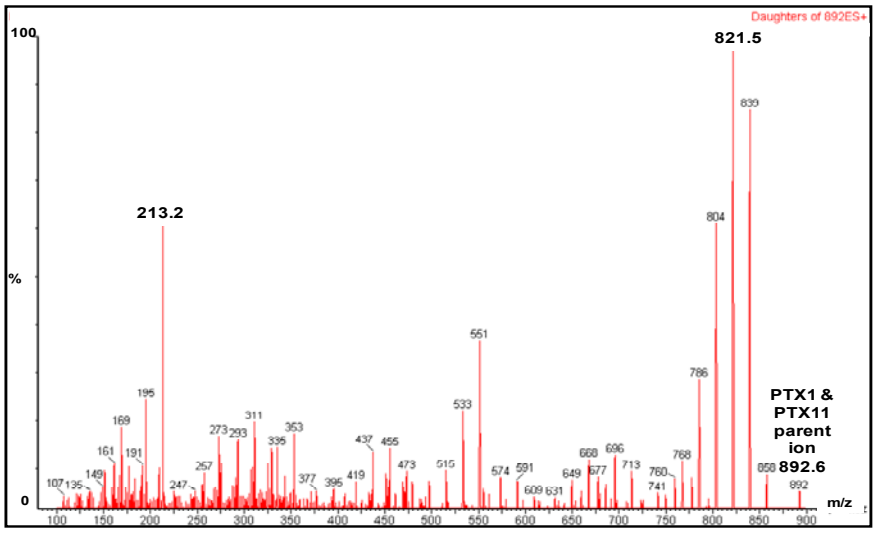
SPX1 and GYM – non regulated lipophilic toxins

Appendix 3. Fragment ion spectra of okadaic acid (OA) and of its isomer, dinophysistoxin 2 (DTX2).

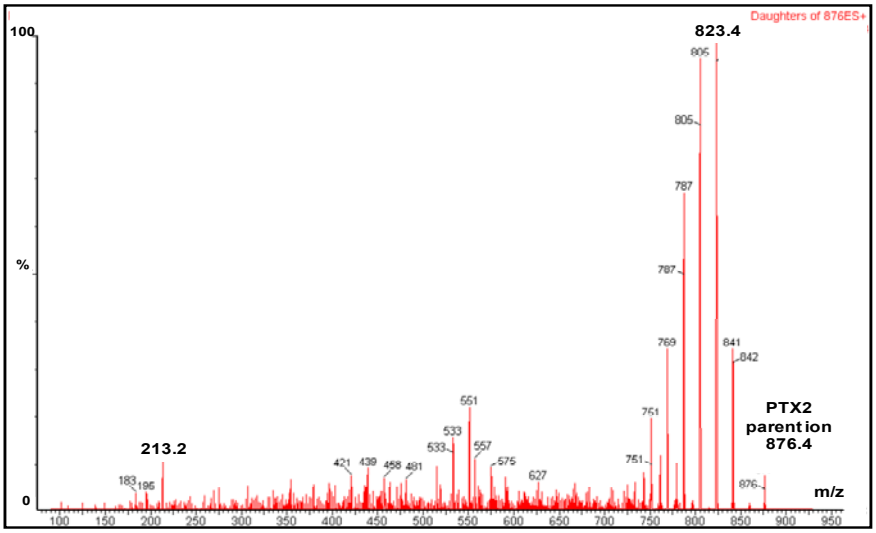


Appendix 4. Fragment ion spectra of Dinophysistoxin 1 (DTX1).

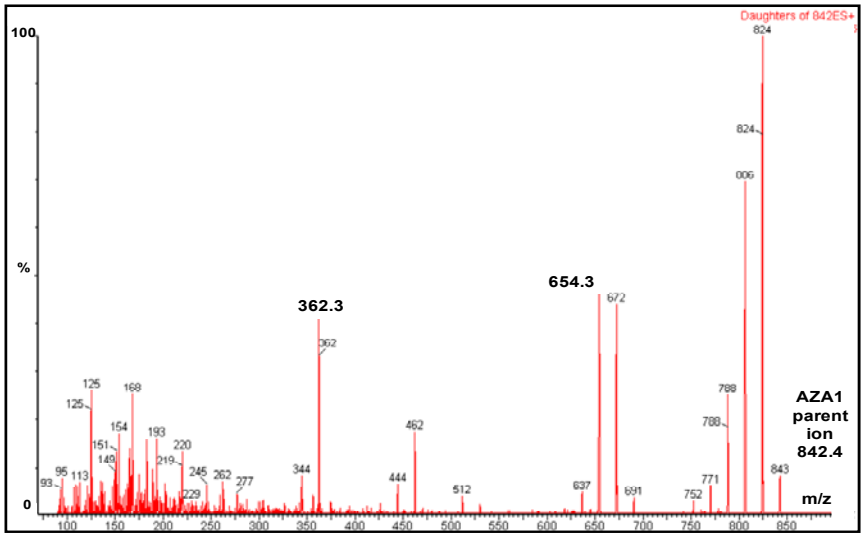
Appendix 5. Fragment ion spectra of Pectenotoxin 1 and its isomer, Pectenotoxin 11 (PTX11).



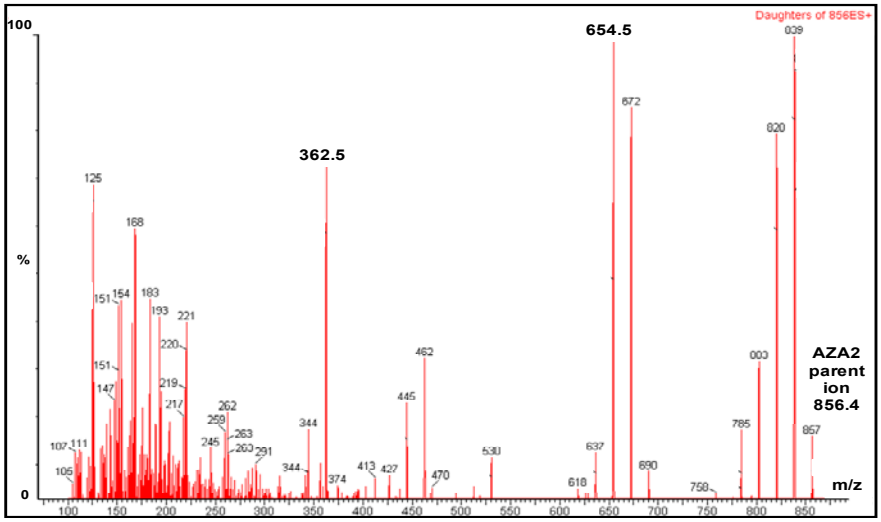
Appendix 6. Fragment ion spectra of Pectenotoxin 2 (PTX2).



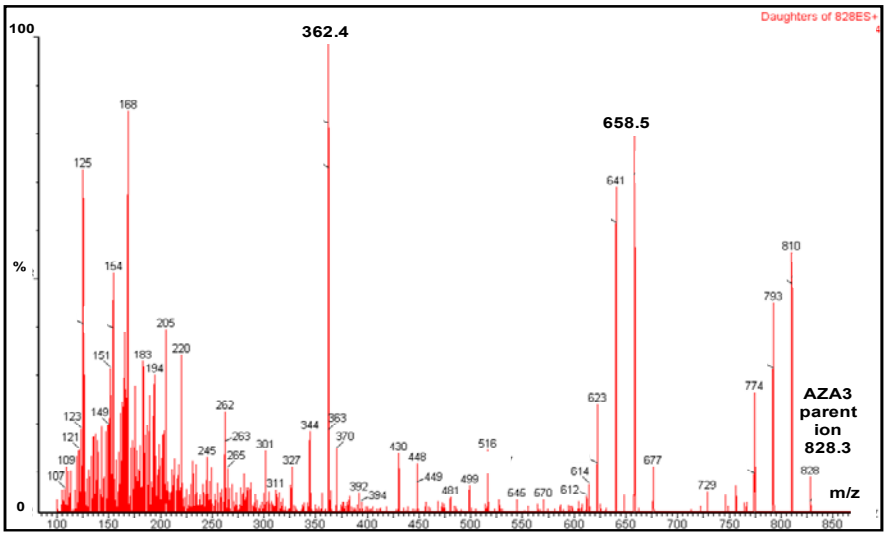
Appendix 7. Fragment ion spectra of Azaspiracid 1 (AZA1).



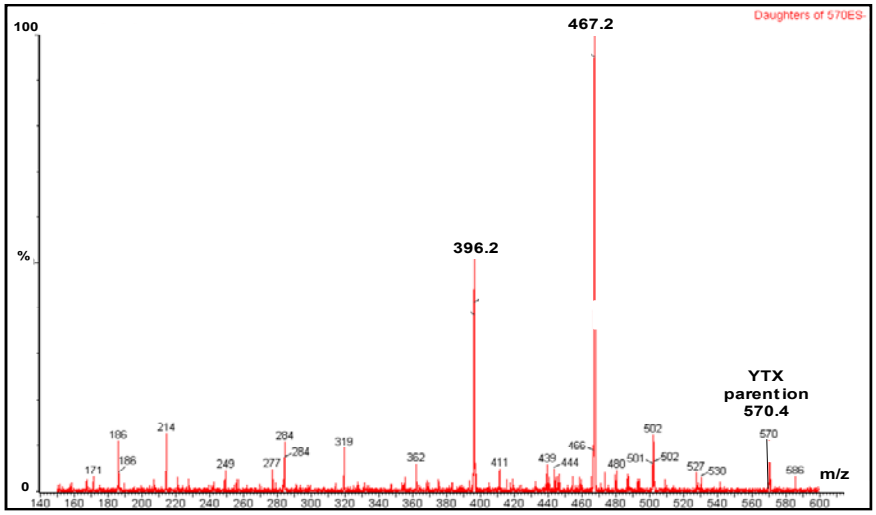
Appendix 8. Fragment ion spectra of Azaspiracid 2 (AZA2).



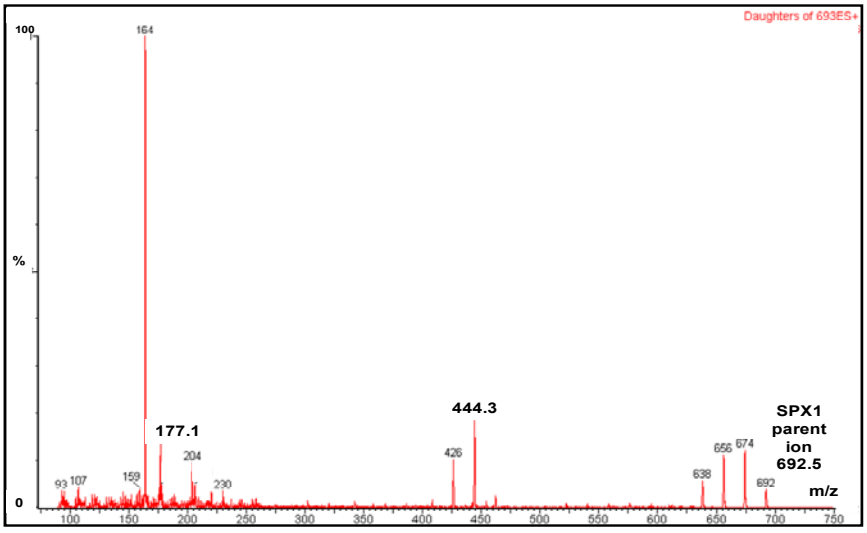
Appendix 9. Fragment ion spectra of Azaspiracid 3 (AZA3).



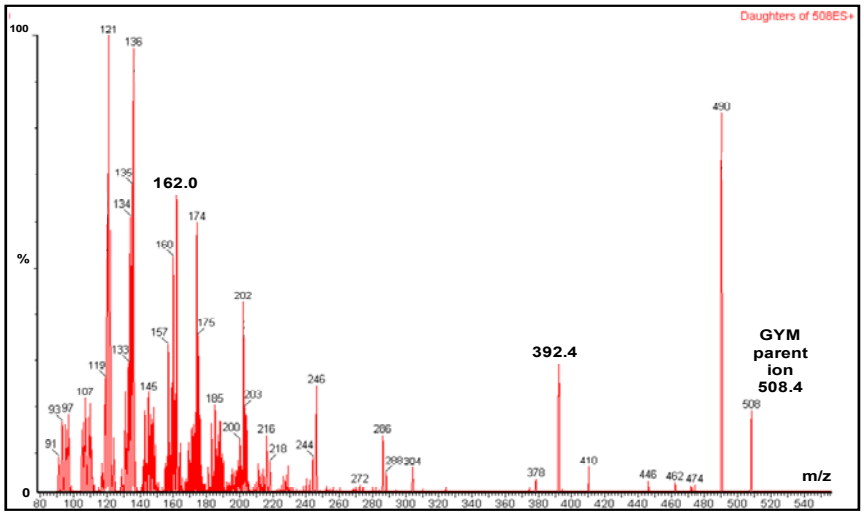
Appendix 10. Fragment ion spectra of Yessotoxin (YTX; $[M-2H]^{2-}$ parent ion m/z 570.0).



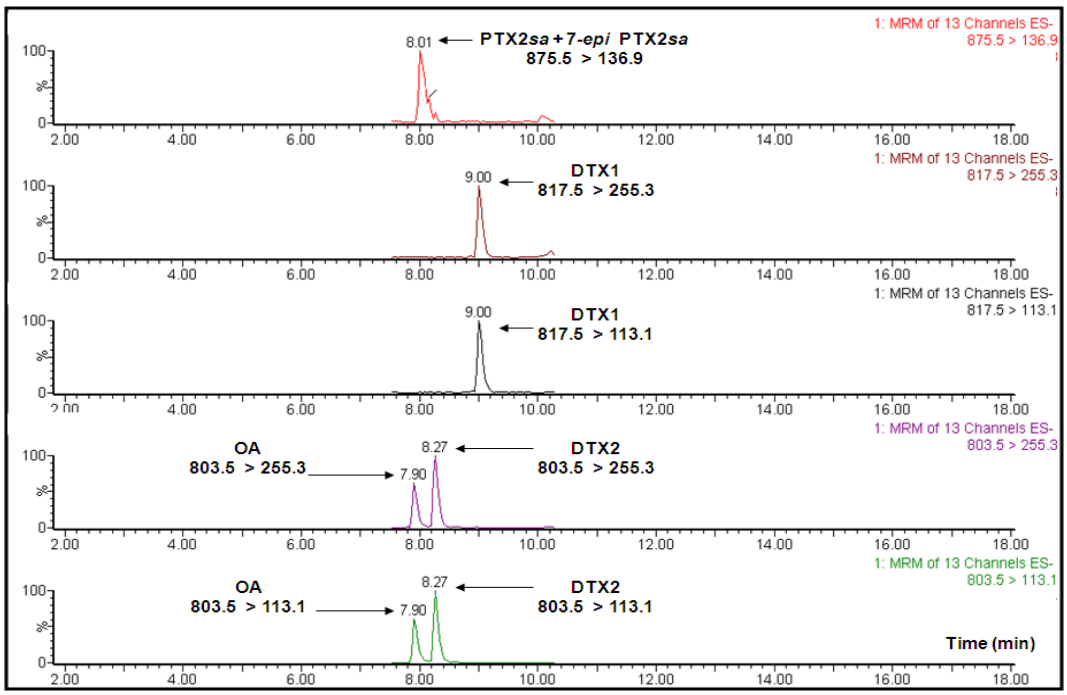
Appendix 11. Fragment ion spectra of 13-desmethyl spirolide C (SPX1).



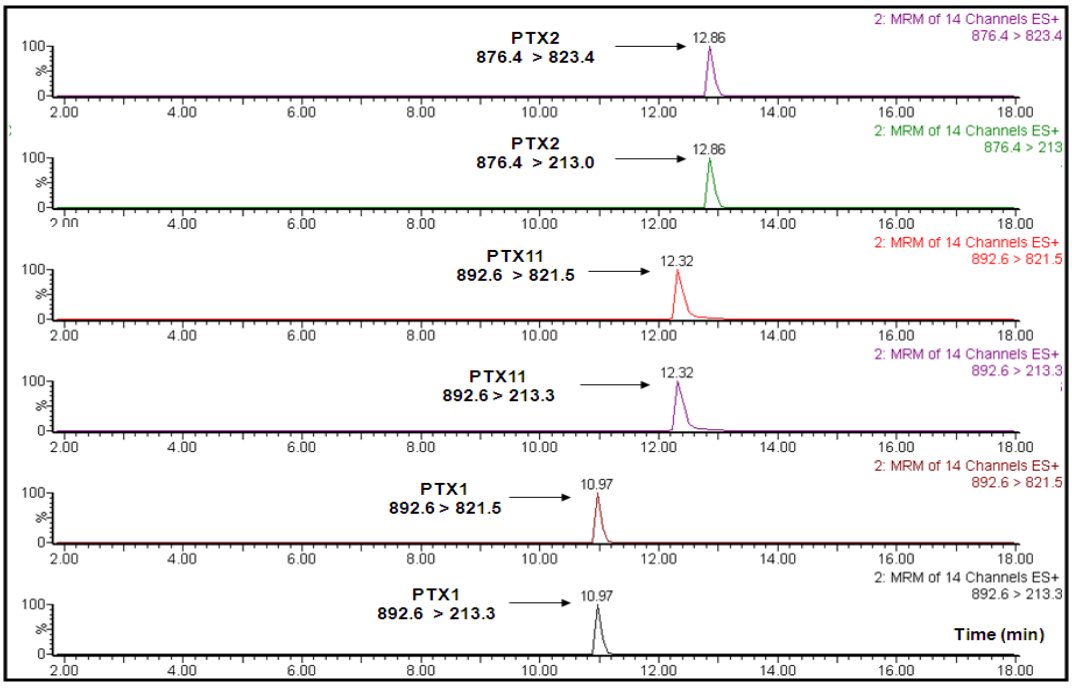
Appendix 12. Fragment ion spectra of Gymnodimine (GYM).



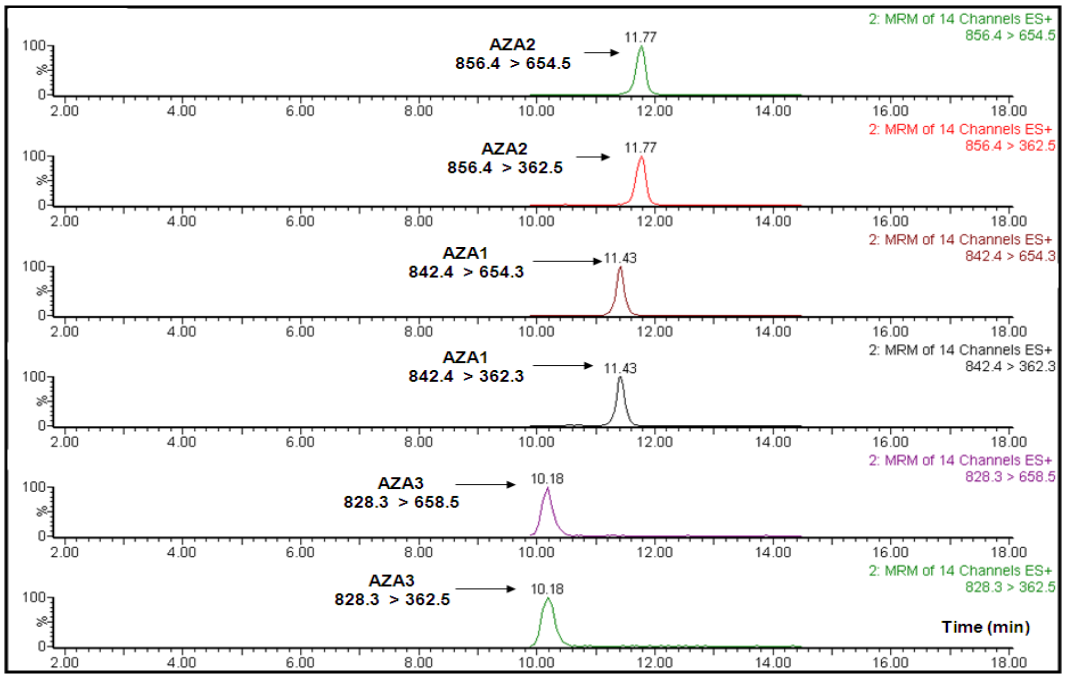
Appendix 13. HPLC elution of okadaic acid (OA), dinophysistoxins 1 and 2 (DTX1, DTX2) and the pectenotoxin 2 metabolite, PTX2*sa*+7-*epi*-PTX2*sa* using the alkaline (pH 11) LC gradient.



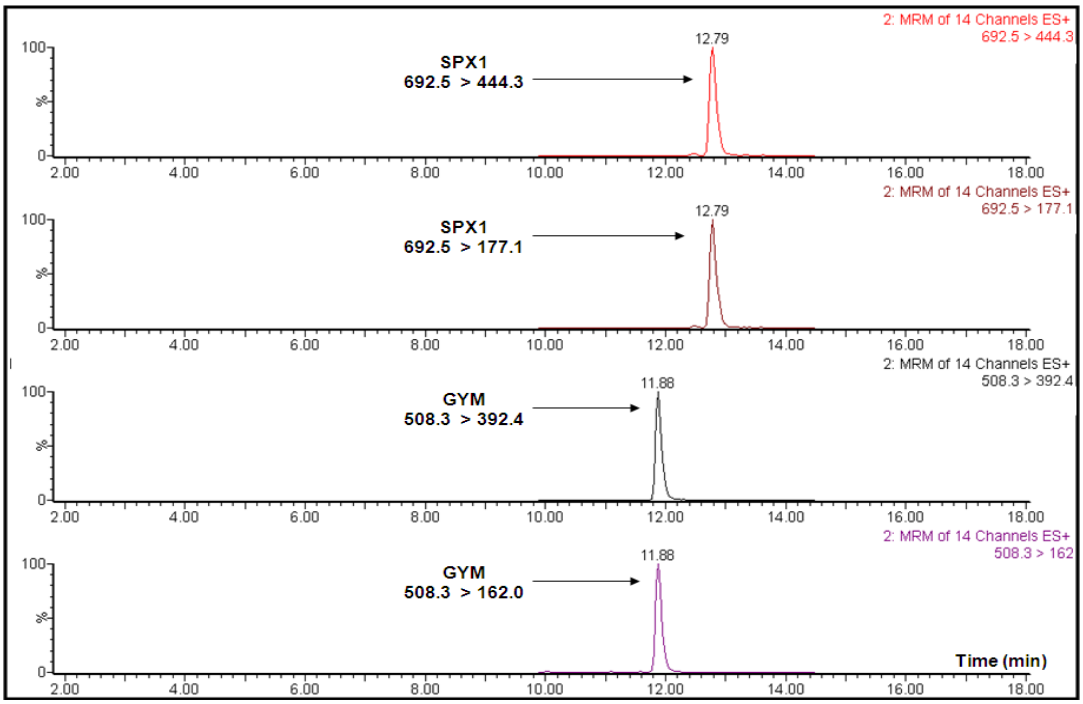
Appendix 14. HPLC elution of pectenotoxins 1, 11 (isomer of PTX1) and 2 (PTX1, 11 and 2) using the alkaline (pH 11) LC gradient.



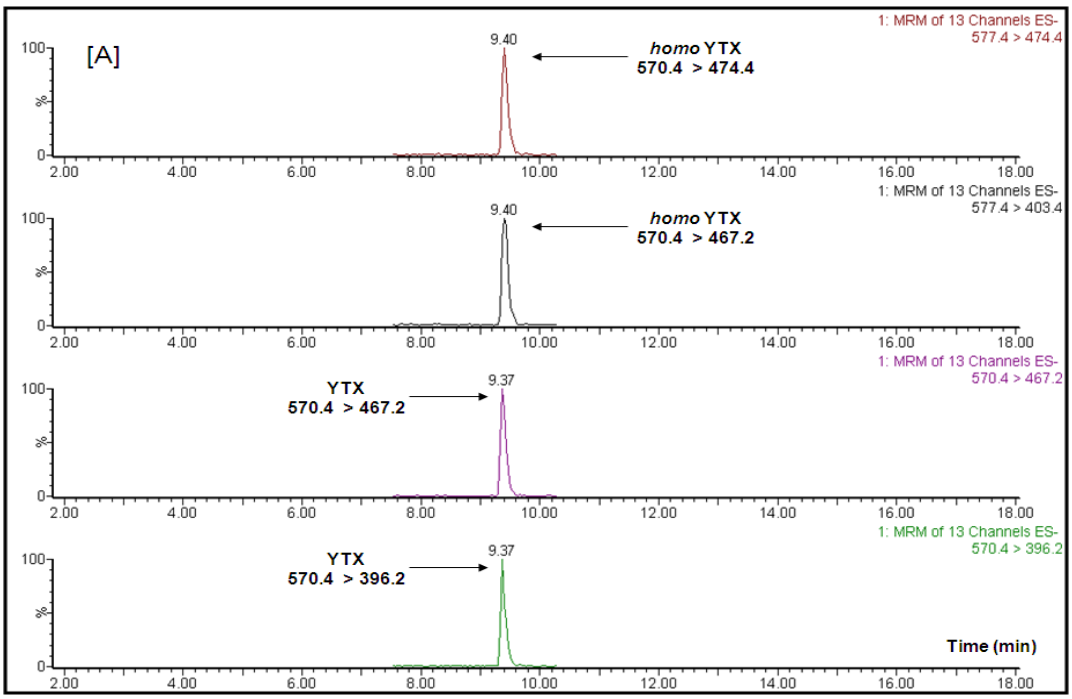
Appendix 15. HPLC elution of azaspiracids 1, 2 and 3 (AZA1/2/3) using the alkaline (pH 11) gradient.

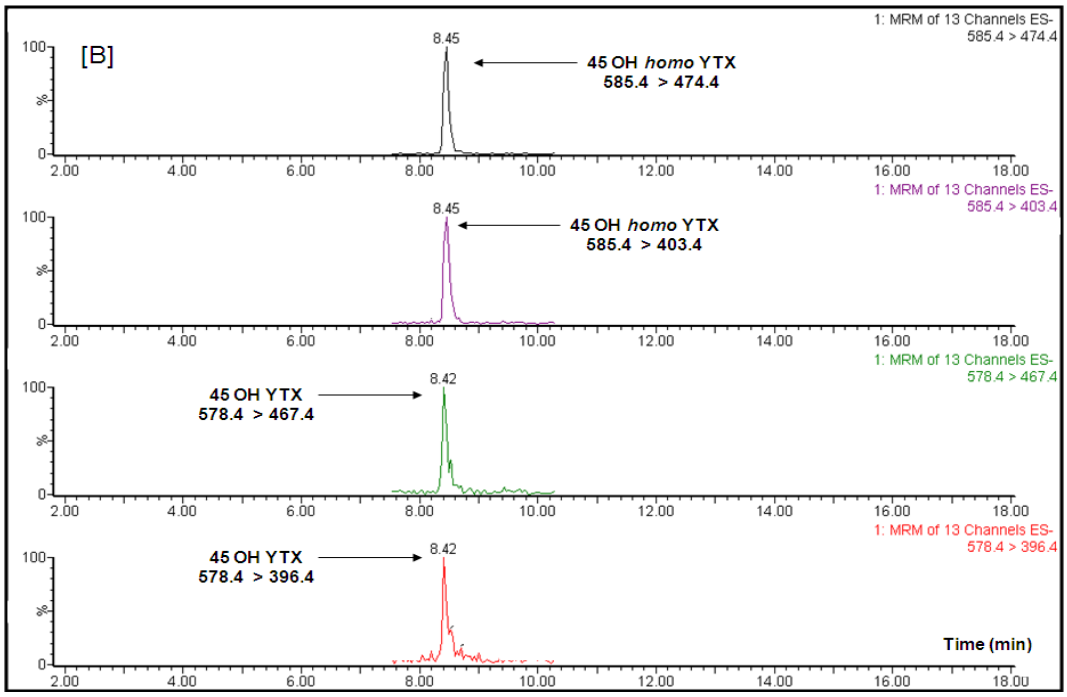


Appendix 16. HPLC elution of the non-regulated cyclic imine toxins - gymnodimine (GYM) and 13-desmethyl spirolide C (SPX1) using the alkaline (pH11) LC gradient.

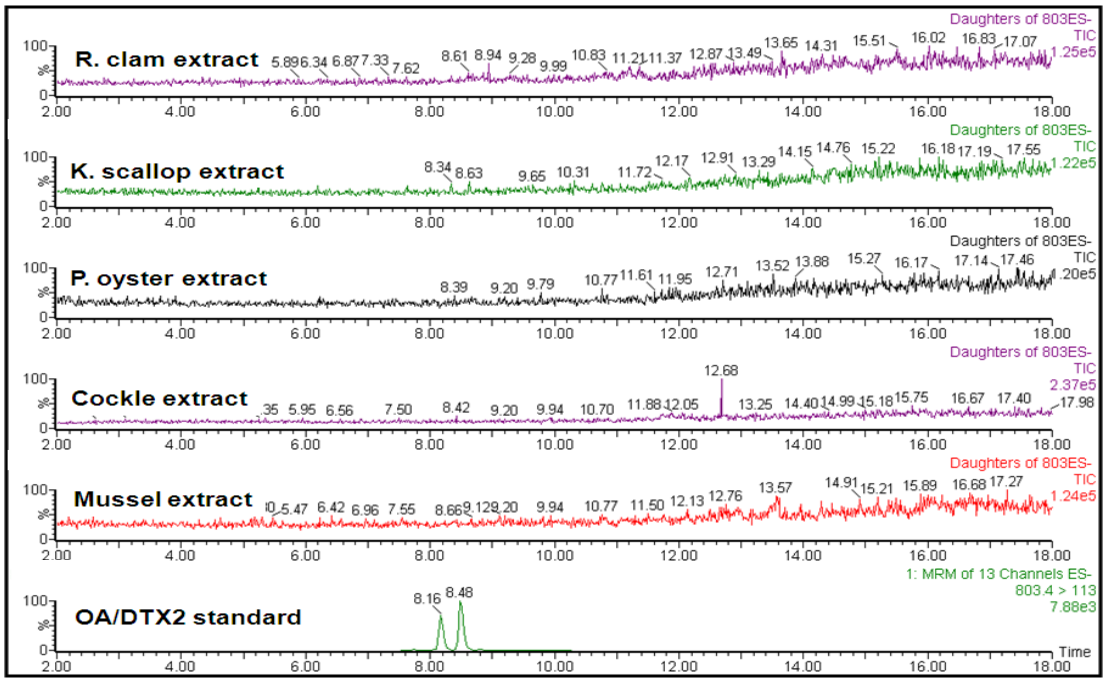


Appendix 17. HPLC elution of [A] YTX and *homo* YTX and [B] 45 OH YTX and 45 OH *homo* YTX using the alkaline (pH11) LC gradient.

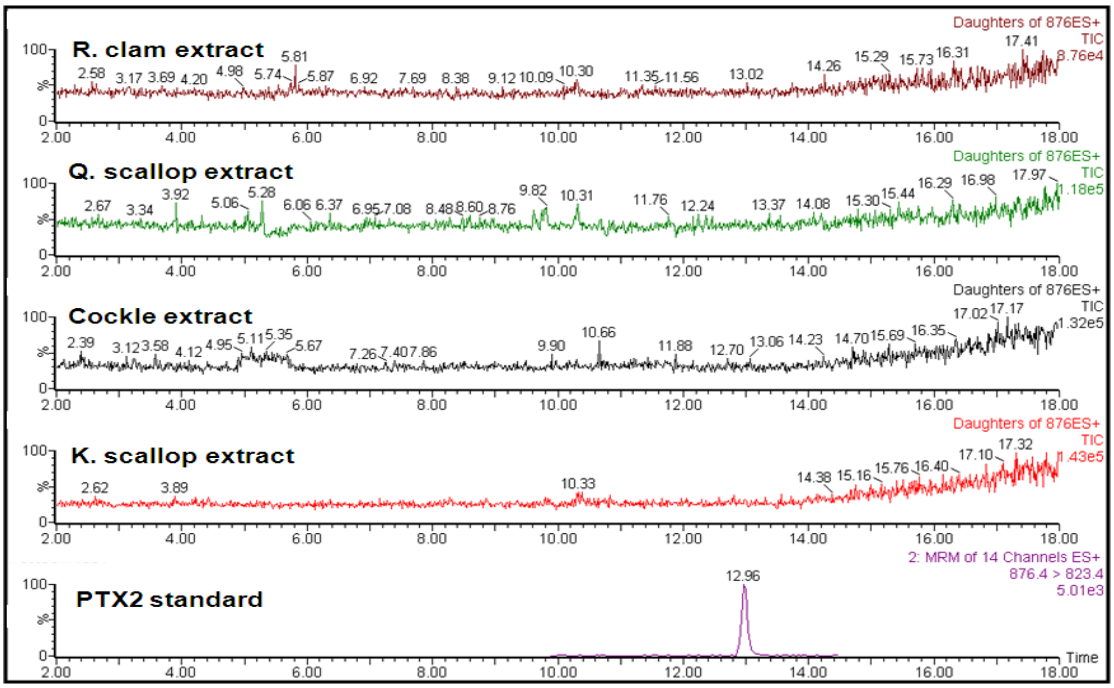




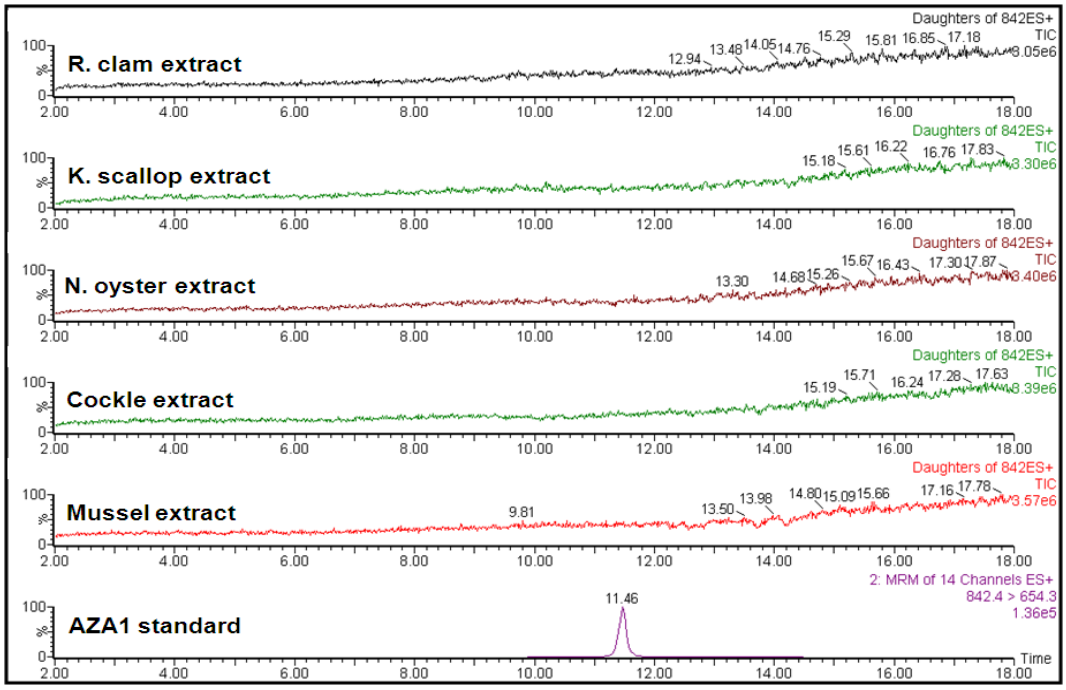
Appendix 18. Comparison of total ion count (TIC) chromatograms of OA and DTX2-free (blank) shellfish extracts with MRM chromatogram of OA and DTX2 (803.4>113.0) reference standards prepared in Common mussel extract.



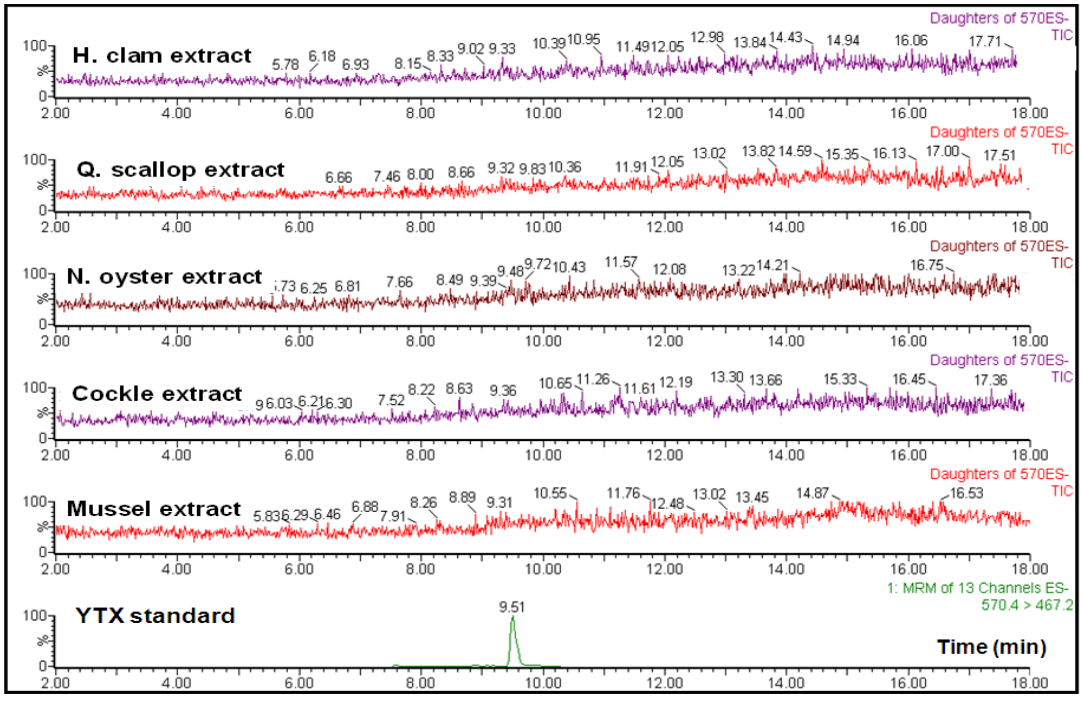
Appendix 19. Comparison of total ion count (TIC) chromatograms of PTX2-free (blank) shellfish extracts with MRM chromatogram of PTX2 (876.5>823.4) reference standard prepared in Common mussel extract.



Appendix 20. Comparison of total ion count (TIC) chromatograms of AZA1-free (blank) shellfish extracts with MRM chromatogram of AZA1 (842.4>654.3) reference standard prepared in Common mussel extract.



Appendix 21. Comparison of total ion count (TIC) chromatograms of YTX-free (blank) shellfish extracts with MRM chromatogram of YTX (570.4>467.2) reference standard prepared in Common mussel extract.



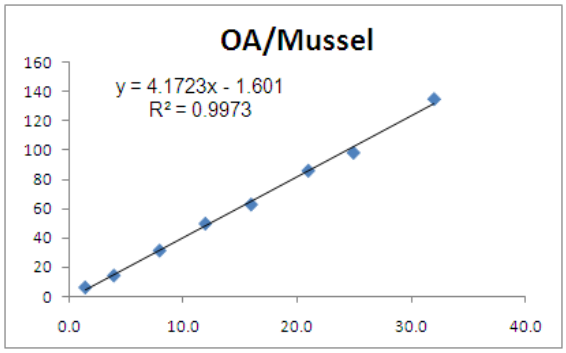
Appendix 22. Calibration coefficients (r^2) of the LC-MS/MS method using the *Quattro Micro* instrument.

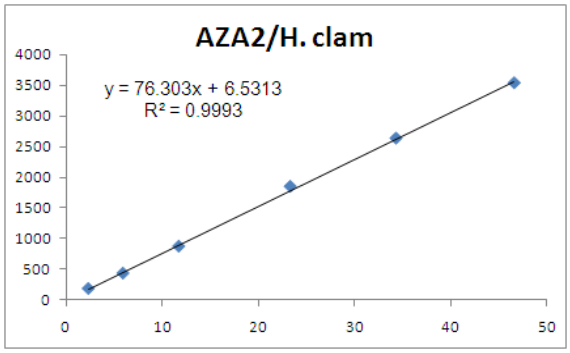
Matrix/ toxin	r^2	Matrix/ toxin	r^2	Matrix/ toxin	r^2
Methanol		**Pacific oyster**		**King scallop**	
OA	0.997	**OA**	0.990	**OA**	0.998
DTX1*	0.999	**DTX1***	0.999	**DTX1***	0.999
DTX2*	0.997	**DTX2***	0.999	**DTX2***	0.998
PTX2	0.999	**PTX2**	0.999	**PTX2**	0.996
PTX11**	0.999	**PTX11****	0.998	**PTX11****	0.998
AZA1	0.999	**AZA1**	0.999	**AZA1**	0.994
AZA2*	0.999	**AZA2***	0.999	**AZA2***	0.999
AZA3*	0.998	**AZA3***	1.000	**AZA3***	0.998
YTX	0.991	**YTX**	0.995	**YTX**	0.999
SPX1	0.997	**SPX1**	0.999	**SPX1**	0.998
GYM	0.999	**GYM**	0.998	**GYM**	0.994
Common mussel		**Native oyster**		**Hard clam**	
OA	0.997	**OA**	0.999	**OA**	0.997
DTX1*	0.999	**DTX1***	0.999	**DTX1***	0.999
DTX2*	0.997	**DTX2***	0.999	**DTX2***	0.999
PTX2	0.998	**PTX2**	0.998	**PTX2**	0.998
PTX11**	0.999	**PTX11****	0.999	**PTX11****	0.999
AZA1	0.999	**AZA1**	0.999	**AZA1**	0.999
AZA2*	0.997	**AZA2***	0.999	**AZA2***	0.999
AZA3*	0.999	**AZA3***	0.997	**AZA3***	0.999
YTX	0.999	**YTX**	0.999	**YTX**	0.999
SPX1	0.998	**SPX1**	0.997	**SPX1**	0.998
GYM	0.998	**GYM**	0.999	**GYM**	0.998
Common cockle		**Queen scallop**		**Razor clam**	
OA	0.998	**OA**	0.994	**OA**	0.998
DTX1*	0.998	**DTX1***	0.999	**DTX1***	0.999
DTX2*	0.999	**DTX2***	0.997	**DTX2***	0.998
PTX2	0.996	**PTX2**	0.998	**PTX2**	0.998
PTX11**	0.999	**PTX11****	0.998	**PTX11****	0.996
AZA1	0.998	**AZA1**	0.998	**AZA1**	0.999
AZA2*	0.999	**AZA2***	0.999	**AZA2***	0.998
AZA3*	0.999	**AZA3***	0.999	**AZA3***	0.996
YTX	0.991	**YTX**	0.999	**YTX**	0.999
SPX1	0.997	**SPX1**	0.997	**SPX1**	0.998
GYM	0.997	**GYM**	0.984	**GYM**	0.999

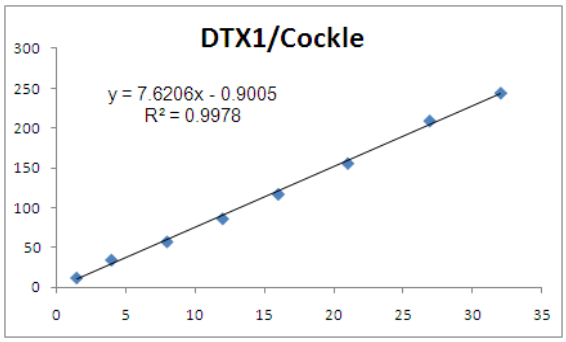
***** Pre-certified reference standards

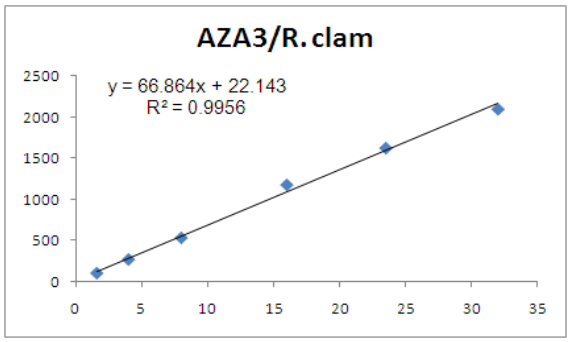
****** Non-certified reference standard

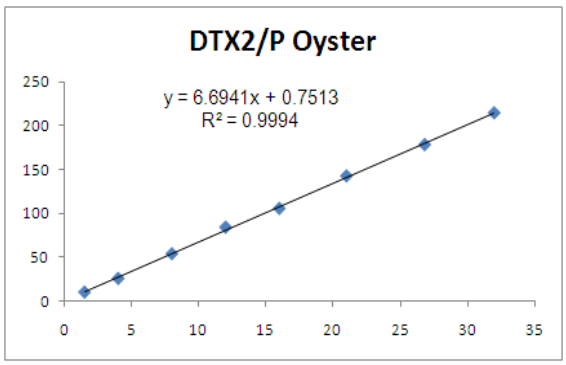
Appendix 23. Examples of linear regression plots of selected toxins in shellfish matrix matched calibration standards.

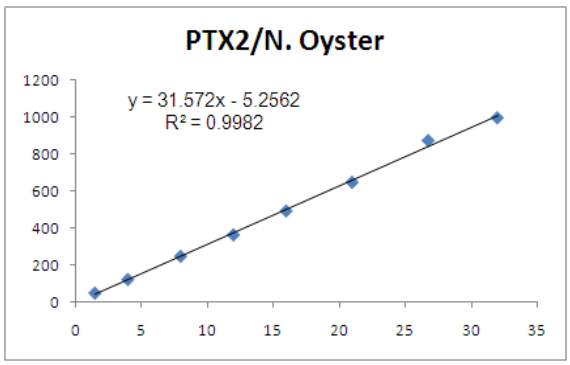


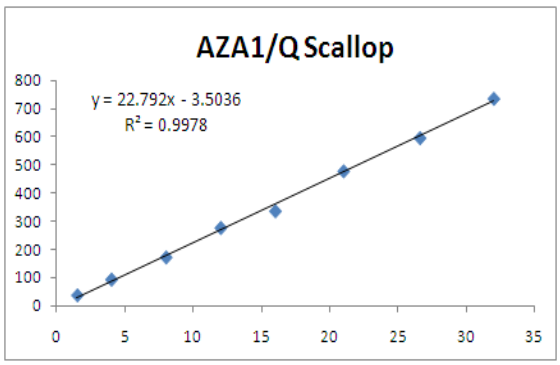


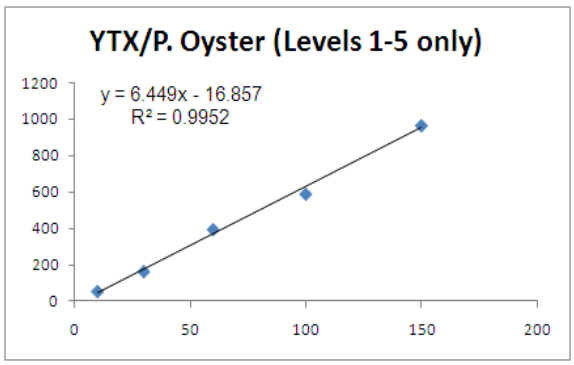












Appendix 24. T-test results (two-tailed; n=7; 95% confidence; t-critical = 2.447) from ruggedness testing of the HPLC-MS/MS method for each shellfish species.

Toxin	Shellfish species	Extraction volume	1st extraction vortex mixing time	Centrifuge speed	2nd extraction mixing speed	Centrifuge time	LC flow rate	pH of mobile phases
OA	Mussel	0.44	-0.24	-0.61	0.30	0.17	-0.42	0.54
	Cockle	-0.48	-0.31	-0.30	0.22	0.38	-0.87	0.66
	Native oyster	0.57	-0.64	0.48	0.26	0.21	-0.25	0.35
	Pacific oyster	-0.63	0.00	-0.03	0.40	-0.08	0.11	-0.82
	King scallop	-0.04	-0.34	0.05	0.28	-0.18	0.10	-0.27
	Queen scallop	0.16	-0.29	-0.27	-0.61	-0.22	0.10	0.70
	Hard clam	1.62	-0.33	-0.16	-1.34	-1.27	-0.19	1.69
	Razor clam	0.05	-0.43	-0.18	-0.10	-0.11	-0.15	-0.06
DTX1	Mussel	0.90	-1.12	-0.99	-0.40	-0.41	0.18	1.36
	Cockle	0.12	-0.27	-0.24	0.29	-0.02	-0.13	0.36
	Native oyster	0.21	-0.22	-0.24	0.12	-0.10	-0.12	-0.03
	Pacific oyster	-0.17	-0.24	0.25	0.52	0.22	-0.02	-0.85
	King scallop	-0.84	0.21	1.39	-0.57	0.39	-0.52	-0.18
	Queen scallop	0.08	-0.08	-0.04	-0.15	0.04	0.27	0.11
	Hard clam	0.60	-0.38	-0.50	0.34	-0.68	0.34	0.56
	Razor clam	0.45	-0.33	-0.32	-0.02	-0.32	-0.11	0.14
DTX2	Mussel	0.08	-0.03	-0.03	0.24	0.16	-0.57	0.70
	Cockle	0.62	-0.12	0.53	0.40	-0.56	-0.03	-0.15
	Native oyster	0.15	0.24	0.42	0.00	0.03	0.28	-0.82
	Pacific oyster	0.37	0.12	1.05	0.67	0.11	-0.82	0.24
	King scallop	-0.26	-0.57	0.15	-0.24	-0.03	0.35	-0.02
	Queen scallop	0.52	0.32	-0.47	-0.15	0.42	0.24	0.04
	Hard clam	1.31	-0.12	-0.20	0.27	0.09	0.44	0.26
	Razor clam	0.30	-0.26	-0.43	-0.22	-0.40	-0.07	0.91
PTX2	Mussel	-0.39	-1.35	0.84	-0.18	-0.39	-0.27	-0.49
	Cockle	0.52	-1.16	0.55	-1.24	-0.12	-0.73	0.18
	Native oyster	-1.79	-1.43	1.11	0.13	-0.43	0.54	-1.30
	Pacific oyster	-1.63	-2.18	1.37	0.09	-0.06	0.27	-2.13
	King scallop	-0.80	0.44	2.12	0.88	-0.56	0.76	-0.34
	Queen scallop	1.02	-2.25	-0.04	-0.34	-1.29	-0.55	0.61
	Hard clam	0.32	-2.08	0.16	-0.04	-1.40	0.02	0.46
	Razor clam	1.55	-0.86	0.81	-1.30	-0.93	-0.68	0.59
AZA1	Mussel	0.06	-0.30	0.10	-0.03	-0.10	0.18	0.14
	Cockle	-0.11	-0.27	0.71	-0.39	-0.15	-0.59	-0.21
	Native oyster	0.02	0.21	-0.05	0.27	0.24	-0.01	0.00
	Pacific oyster	-0.38	-0.18	0.34	0.34	-0.05	0.31	-0.62
	King scallop	-0.06	-0.59	2.32	-0.88	1.10	-0.46	0.24
	Queen scallop	0.96	-0.27	-0.20	0.09	-0.34	-0.24	1.80
	Hard clam	0.55	-0.30	-0.25	-0.03	-0.18	0.04	1.01
	Razor clam	-0.07	-0.76	1.03	-0.68	-0.22	-0.08	-0.53
YTX	Mussel	-1.15	1.08	0.36	-0.45	0.29	-1.37	0.86
	Cockle	-0.49	0.28	0.18	-0.11	-0.14	-0.25	-0.94
	Native oyster	-0.20	1.03	0.58	-0.19	-0.15	0.23	0.60
	Pacific oyster	-0.64	-0.44	0.30	0.29	0.20	-0.58	0.80
	King scallop	-0.19	1.62	-0.15	-0.06	-0.72	-0.44	-0.24
	Queen scallop	2.23	-0.20	-2.03	0.29	2.43	-1.69	1.71
	Hard clam	1.06	0.69	-0.34	-1.46	1.10	0.72	2.25
	Razor clam	-0.01	0.62	0.19	-0.41	-0.91	0.31	-0.18

Appendix 25. Optimised *Xevo TQ* (Waters Ltd., UK) mass spectrometric source cone voltages and collision energies (CE), and relative ion intensities (of base, quantitation ion peak; highlighted) for the production of precursor and transition (fragment) ions of regulated and non-regulated marine lipophilic toxins.

Toxin	MW (amu)	Ionisation mode	Pseudo-molecular ion	Precursor Ion m/z	Transition Ions m/z	Cone (V)	CE (eV)	Relative ion intensity (% of base peak)
OA	804.5	-ve	$[M-H]^-$	803.6	113.0	80	57	35
					255.1	90	46	
DTX1	818.5	-ve	$[M-H]^-$	817.6	113.0	85	58	29
					255.1	85	49	
DTX2	804.5	-ve	$[M-H]^-$	803.6	113.0	80	57	37
					255.1	90	46	
PTX1	876.5	+ve	$[M+NH_4]^+$	892.5	213.1	34	40	40
					821.5	34	26	
PTX11*	876.5	+ve	$[M+NH_4]^+$	892.5	213.1	34	40	47
					821.5	34	26	
PTX2	858.5	+ve	$[M+NH_4]^+$	876.4	213.1	37	40	43
					823.5	37	26	
AZA1	841.5	+ve	$[M+H]^+$	842.6	362.3	63	52	81
					654.4	63	49	
AZA2	855.5	+ve	$[M+H]^+$	856.6	362.3	63	52	73
					654.4	63	50	
AZA3	827.5	+ve	$[M+H]^+$	828.6	362.3	63	52	83
					658.4	63	49	
YTX	1142.5	-ve	$[M-2H]^{2-}$	570.5	396.3	55	38	24
					467.3	55	32	
***homo* YTX**	1156.5	-ve	$[M-2H]^{2-}$	577.5	403.3	55	38	21
					474.3	55	32	
45 OH YTX	1158.5	-ve	$[M-2H]^{2-}$	578.5	396.3	55	38	29
					467.3	55	32	
45 OH *homo* YTX	1172.5	-ve	$[M-2H]^{2-}$	585.5	403.3	55	38	29
					474.3	55	32	
GYM	507.5	+ve	$[M+H]^+$	508.4	136.1	37	36	28
					162.1	37	42	
SPX1	691.5	+ve	$[M+H]^+$	692.5	164.1	50	49	65
					444.3	50	37	

* isomer of PTX1
MW = molecular weight
amu = (mono-isotopic) atomic mass unit
eV = electron volts
V = volts
MS (data acquisition) file
Dwells 19 to 46 ms
Polarity/mode switch inter-scan delay = 0.02 s
MS Inter-scan delay = 0.005 s

SPX1 and GYM – non regulated lipophilic toxins

Appendix 26. Performance of the LC-MS/MS method in the determination of OA and DTX toxins in QUASIMEME exercise (Round 55; April to July 2009).

Sample type	Sample no.	Toxin	Cefas value (µg/kg)	Assigned mean (µg/kg)	*n*	RSD_R	Cefas z-score	
Standard solution	62	free OA	71.8	68.0	17	13.3	0.4	S
Scallop tissue (species not defined)	63	free OA	243.9	246.2	16	13.4	0.1	S
		Σfree OA/DTXs	243.9	232.6	12	14.2	0.4	S
		Tot. OA	2172	1832	14	16.5	1.5	S
		ΣTot. OA/DTXs	2172	1897	13	14.8	1.2	S
Clam tissue (species not defined)	64	free OA	75.7	71.0	17	24.8	0.5	S
		free DTX1	95.2	97.2	17	30.2	-0.2	S
		free DTX2	214.7	215	15	22.8	0.3	S
		Σfree OA/DTXs	379.9	394.5	15	19.7	0.3	S
		Tot. OA	439.8	396.9	15	24.3	0.9	S
		Tot. DTX1	113.2	113.0	15	31.8	0.1	S
		Tot. DTX2	590.9	601.4	14	27.6	0.1	S
		ΣTot. OA/DTXs	1156	1115	13	16.0	0.3	S
Oyster tissue (species not defined)	65	free OA	5.80	15.31	10	18.8	**-4.8**	**U**
		free DTX1	359.5	284.4	16	23.8	**2.1**	**Q**
		Σfree OA/DTXs	365.3	301.9	14	22.0	1.7	S
		Tot. OA	27.5	30.71	14	16.2	-0.8	S
		Tot. DTX1	453.2	413.3	14	19.0	0.8	S
		ΣTot. OA/DTXs	435.5	480.6	14	17.5	0.8	S

n = number of participants RSD_R = between laboratory precision
S = satisfactory Q = questionable U = unsatisfactory
Σfree OA/DTXs = sum of free OA and DTXs from unhydrolysed extract
ΣTot. OA/DTXs = sum of total (free + esters) OA, total DTX1 and total DTX2 (each toxin determined after extract hydrolysis).

Appendix 27. Performance of the LC-MS/MS method in the determination of OA, DTX and AZA toxins in QUASIMEME exercise (Round 59; October 2009 to January 2010).

Sample Type	Sample no.	Toxin	Cefas value (µg/kg)	Assigned mean (µg/kg)	*n*	RSD_R	Cefas z-score	
Standard solution	80	AZA1	16.0	18.3	15	12.7	-1.0	S
Standard solution	81	AZA1	12.4	13.5	15	13.5	-0.6	S
Shellfish extract (species not defined)	82	free OA	14.9	15.1	14	20.3	-0.1	S
		free DTX1	2.70	2.22	7	30.1	1.5	S
		Σfree OA/DTXs	19.9	17.8	12	21.7	0.9	S
		Tot. OA	19.9	18.0	12	20.3	0.8	S
		ΣTot. OA/DTXs	26.4	22.3	12	22.3	1.5	S
		AZA1	37.1	42.6	15	14.3	-1.0	S
		AZA2	7.10	9.70	15	35.8	**-2.1**	**Q**
		AZA3	8.70	12.3	15	35.8	**-2.3**	**Q**
		ΣAZAs	52.9	67.1	15	21.3	-1.7	S
Scallop (*Pecten maximus*)	83	free OA	276.9	256.9	15	16.8	0.6	S
		Σfree OA/DTXs	288.3	260.8	14	18.1	0.8	S
		Tot. OA	2752	1949	14	29.0	**3.3**	**U**
		ΣTot. OA/DTXs	2780	1950	14	29.0	**3.4**	**U**
		AZA1	120.5	126.7	15	12.4	-0.4	U
		AZA2	25.5	29.8	14	21.4	-1.1	S
		AZA3	29.0	42.1	14	33.5	**-2.5**	**Q**
		ΣAZAs	175	204.6	14	20.5	-1.2	S
Mussel (*Mytilus edulis*)	84	free OA	227.4	206.9	16	14.4	0.8	S
		free DTX2	234.8	227.5	15	29.4	0.3	S
		Σfree OA/DTXs	471.9	427.9	14	22.5	0.8	S
		Tot. OA	526.3	395.0	14	24.6	**2.7**	**Q**
		Tot. DTX2	350.5	330.8	14	10.6	0.5	S
		ΣTot. OA/DTXs	887.2	723.8	14	18.8	1.8	S
		AZA1	1178	1227	14	13.6	-0.3	S
		AZA2	234.1	300.1	14	19.5	-1.8	S
		AZA3	166.9	271.6	14	38.2	**-3.1**	**U**
		ΣAZAs	1579	1790	14	15.9	-0.9	S

n = number of participants RSD_R = between laboratory precision
S = satisfactory Q = questionable U = unsatisfactory
Σfree OA/DTXs = sum of free OA and DTXs from unhydrolysed extract
ΣTot. OA/DTXs = sum of total (free + esters) OA, total DTX1 and total DTX2 (each toxin determined after extract hydrolysis).

Appendix 28. Performance of the LC-MS/MS method of the determination of OA, DTX and AZA toxins in QUASIMEME exercise (Round 61; April to August 2010).

Sample Type	Sample no.	Toxin	Cefas value (µg/kg)	Assigned mean (µg/kg)	*n*	RSD_R	Cefas z-score	
Standard solution	88	free OA	67.4	67.8	19	27.2	0.0	S
		Σfree OA/DTXs	67.4	69	15	22.5	-0.2	S
Standard solution	89	AZA1	14.3	15.4	19	28.5	-0.6	S
		ΣAZAs	14.3	15.7	16	24.8	-0.7	S
Shellfish extract (species not defined)	90	free OA	12.3	13.6	17	17.6	-0.8	S
		free DTX2	24.6	28.1	17	18.1	-1.0	S
		Σfree OA/DTXs	36.9	40.4	16	19.5	-0.7	S
		Tot. OA	43.8	49.1	14	42.9	-0.9	S
		Tot. DTX2	49.5	60.3	14	54.1	-1.4	S
		ΣTot. OA/DTXs	93.3	109.9	14	47.5	-1.2	S
		AZA1	13.7	17.1	19	17.5	-1.5	S
		AZA2	3.00	4.29	14	43.4	**-2.2**	**Q**
		AZA3	3.90	5.59	15	44.8	**-2.3**	**Q**
		ΣAZAs	20.6	25.6	17	31.4	-1.5	S
Mussel (*Mytilus edulis*)	91	free OA	97.3	134.1	17	26.7	**-2.2**	**Q**
		free DTX1	88.4	89.4	18	36.5	-0.1	S
		free DTX2	391.7	579.6	17	32.8	**-2.6**	**Q**
		Σfree OA/DTXs	577.4	804	16	28.0	**-2.3**	**Q**
		Tot. OA	353.7	328.3	15	35.0	0.6	S
		Tot. DTX1	129.5	134.3	15	32.9	-0.3	S
		Tot. DTX2	724.9	889.7	15	25.0	-1.5	S
		ΣTot. OA/DTXs	1208	1345	15	28.4	-0.8	S
		AZA1	673.1	897.9	18	17.7	**-2.0**	**Q**
		AZA2	157.8	238.5	17	28.7	**-2.7**	**Q**
		AZA3	130.6	204.6	17	28.6	**-2.9**	**Q**
		ΣAZAs	961.5	1310	17	22.9	**2.1**	**Q**
Mussel (*Mytilus edulis*)	92	free OA	212.4	212.5	18	15.4	0.0	S
		free DTX2	195.0	253.1	17	30.5	-1.8	S
		Σfree OA/DTXs	407.4	478.0	16	25.0	-1.2	S
		Tot. DTX1	410.3	395.1	15	26.2	0.3	S
		Tot. DTX2	268.7	353.2	15	34.7	-1.9	S
		ΣTot. OA/DTXs	679.0	724.5	15	32.8	-0.5	S
		AZA1	1141	1231	18	21.4	-0.6	S
		AZA2	238.2	310.3	17	25.8	-1.9	S
		AZA3	180.0	261.3	17	16.3	**-2.5**	**Q**
		ΣAZAs	1560	1787	17	20.1	-1.0	S

n = number of participants RSD_R = between laboratory precision
S = satisfactory Q = questionable U = unsatisfactory
Σfree OA/DTXs = sum of free OA and DTXs from unhydrolysed extract
ΣTot. OA/DTXs = sum of total (free + esters) OA, total DTX1 and total DTX2 (each toxin determined after extract hydrolysis).

Appendix 29. Certificate of Cefas' performance in BLV (Germany) main collaborative study (2009).

Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Results of the laboratory

Cefas
(Weymouth, UK)

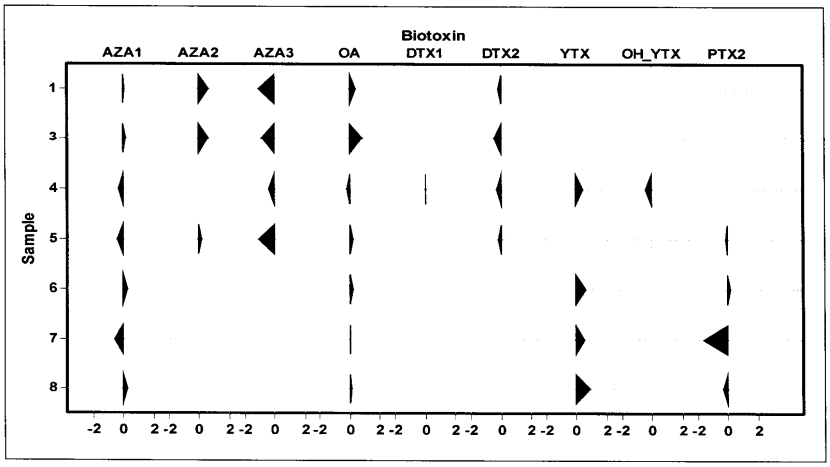
in the collaborative method validation study

"Marine Lipophilic Biotoxins".

The results of this laboratory are presented in the following chart of Z_u scores. A Z_u score smaller than -2 indicates a laboratory mean value that is statistically significantly smaller than the overall mean value, a Z_u score larger than +2 indicates a laboratory mean value that is statistically significantly larger than the overall mean value. Significant Z_u scores are marked in red.

Shown are the Z_u scores for all nine analytes in seven test samples. Samples 1, 3 and 4 are cooked homogenates of blue mussels. Samples 1 and 3 are blind duplicates. Sample 5 is an extract of samples 1 and 3. Samples 6 and 8 are raw extracts of oysters and also blind duplicates. Sample 7 is a raw extract of clams.

Please note that only those Z_u scores are shown for which measurement values above the limit of detection were available from at least six laboratories and for which the measurement values of this laboratory were above the limit of detection as well.



Statistical evaluation by quo data
www.quodata.de

Appendix 30. Cefas laboratory mean and assigned mean concentrations (µg/kg), and within (RSD_r) and between (RSR_R) laboratory relative standard deviations and HorRat values for the determination of free OA and DTX toxins obtained in the RIKILT LC-MS/MS collaborative study.

Sample	Matrix	Lab. mean (µg/kg)	Assigned mean (µg/kg)	RSD_r (%)	RSD_R (%)	HorRat value	Nos. of Labs a(b)
Free OA (as determined from OA calibration; r^2 = 1.00; response drift = -3.0%)							
1	Mussel	55.4	47.8	8.3	20.1	0.79	11(0)
2	Mussel	48.7	39.3	15.5	23	0.88	11(0)
3	Mussel	108.5	89.1	12.1	19.9	0.86	11(0)
4	Oyster	151.5	120.7	7.7	16.3	0.74	11(0)
5	Mussel	187.8	160.4	3.4	27.8	1.32	11(0)
6	Mussel	63.3	54.4	8	23	0.93	11(0)
7	Cockle	353	297.7	6.2	15.5	0.81	11(0)
8	Oyster	81.5	69.1	8.4	19.9	0.83	11(0)
Free DTX1 (as determined from DTX1 calibration; r^2 = 1.00; response drift = 3.2%)							
1	Mussel	34.0	36.8	10.9	16.7	0.63	11 (0)
2	Mussel	76.0	69.6	5.6	12.7	0.53	11 (0)
3	Mussel	<LOQ	<LOQ				
4	Oyster	<LOQ	<LOQ				
5	Mussel	67.4	70.7	4.0	12.0	0.5	9 (1)
6	Mussel	<LOQ	<LOQ				
7	Cockle	97.2	106.5	7.2	13.7	0.61	10 (0)
8	Oyster	13.3	17.3	12.3	26.1	0.88	10 (0)
Free DTX1 (as determined from OA calibration; r^2 = 1.00; response drift = -3.0%)							
1	Mussel	46.1	29.5	10.3	27.7	1.02	10(1)
2	Mussel	108.2	64.9	3.9	49.4	2.04	11(0)
3	Mussel	<LOQ	<LOQ				
4	Oyster	<LOQ	<LOQ				
5	Mussel	95.3	58.5	3.9	30.7	1.25	10(1)
6	Mussel	<LOQ	<LOQ				
7	Cockle	139.5	85.4	6.1	31.1	1.34	10(1)
8	Oyster	13.3	13.5	11.2	28.8	0.94	10(1)
Free DTX2 (as determined from DTX2 calibration; r^2 = 1.00; response drift = 7.8%)							
1	Mussel	114.5	111.0	5.9	17.2	0.77	11(0)
2	Mussel	<LOQ	<LOQ				
3	Mussel	79.3	63.5	11.6	18.8	0.78	11(0)
4	Oyster	147.7	142.0	4.5	16.7	0.78	11(0)
5	Mussel	<LOQ	<LOQ				
6	Mussel	16.2	13.5	29.2	36.4	1.19	10(0)
7	Cockle	147.6	144.1	5	14	0.65	10(0)
8	Oyster	104.5	87.7	4.5	15.7	0.68	10(0)
Free DTX2 (as determined from OA calibration; r^2 = 1.00; response drift = -3.0%)							
1	Mussel	143.7	133.1	6.3	13.4	0.62	11(0)
2	Mussel	<LOQ	<LOQ				
3	Mussel	97.3	75.6	13.7	21.5	0.91	11(0)
4	Oyster	187.3	172.9	4.6	21	1.01	11(0)
5	Mussel	<LOQ	<LOQ				
6	Mussel	14.5	14.2	26	32.1	1.06	11(0)
7	Cockle	187.1	171.6	6.3	16.7	0.8	11(0)
8	Oyster	130.5	103.9	4.9	20.7	0.92	10(1)

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

Appendix 31. Cefas laboratory mean and assigned mean concentrations (µg/kg), and within (RSD_r) and between (RSD_R) laboratory relative standard deviations and HorRat values for the determination of total OA and DTX toxins (after hydrolysis) obtained in the RIKILT LC-MS/MS collaborative study.

Sample	Matrix	Lab. mean (µg/kg)	Assigned mean (µg/kg)	RSD_r (%)	RSD_R (%)	HorRat value	Nos. of Labs a(b)
Total OA (as determined from OA calibration; r^2 = 0.98; response drift = 33.2%)							
1	Mussel	131.3	137.8	5.7	23.9	1.11	9(0)
2	Mussel	73.1	66.0	5.6	24.7	1.02	9(0)
3	Mussel	215.6	177.6	12.9	24.9	1.2	9(0)
4	Oyster	231.3	249.1	5.1	27.5	1.4	9(0)
5	Mussel	635.6	618.3	7.3	31.1	1.81	9(0)
6	Mussel	70.6	74.2	10.1	23.5	0.99	9(0)
7	Cockle	996	976.9	29.9	29.2	1.82	9(0)
8	Oyster	238.5	243.3	6.1	22.8	1.15	9(0)
Total DTX1 (as determined from DTX1 calibration; r^2 = 1.00; response drift = -19.5%)							
1	Mussel	57.8	55.3	12.3	24.6	1.0	11(0)
2	Mussel	95.8	98.2	9.7	23.7	1.04	11(0)
3	Mussel	<LOQ	<LOQ				
4	Oyster	<LOQ	<LOQ				
5	Mussel	129.8	137.9	7.0	19.4	0.9	10(0)
6	Mussel	<LOQ	<LOQ				
7	Cockle	177.9	188.4	5.6	17.8	0.87	9(1)
8	Oyster	33.2	33.7	11.1	21.4	0.8	10(0)
Total DTX1 (as determined from OA calibration; r^2 = 0.98; response drift = 33.2%)							
1	Mussel	66.3	36.4	9.4	20.4	0.77	9(0)
2	Mussel	115.9	66.6	7.8	22.1	0.92	9(0)
3	Mussel	<LOQ	<LOQ				
4	Oyster	<LOQ	<LOQ				
5	Mussel	160.3	97.7	5.9	27.8	1.22	8(1)
6	Mussel	<LOQ	<LOQ				
7	Cockle	223.1	132.5	20.2	34.5	1.59	8(1)
8	Oyster	34.2	23.9	11.4	24.5	0.87	8(1)
Total DTX2 (as determined from DTX2 calibration r^2 = 0.99; response drift = 25.9%)							
1	Mussel	180.9	182.1	7.1	20.3	0.98	11(0)
2	Mussel	<LOQ	<LOQ				
3	Mussel	105.8	105.6	15.8	27.3	1.21	11(0)
4	Oyster	223.1	251.5	7.1	27.9	1.42	11(0)
5	Mussel	<LOQ	<LOQ				
6	Mussel	15.6	13.5	41.1	49.4	1.62	11(0)
7	Cockle	216.1	233.3	25.1	29.4	1.47	11(0)
8	Oyster	156.8	160.0	11.9	27.2	1.29	11(0)
Total DTX2 (as determined from OA calibration; r^2 = 0.98; response drift = 33.2%)							
1	Mussel	212.5	189.6	6.3	36.6	1.78	9(0)
2	Mussel	<LOQ	<LOQ				
3	Mussel	123.3	107.1	18.4	35.2	1.57	9(0)
4	Oyster	262.4	260.5	7.3	40.1	2.05	9(0)
5	Mussel	<LOQ	<LOQ				
6	Mussel	16.3	15.7	19.2	45.6	1.52	9(0)
7	Cockle	254.1	261.6	30.5	29.7	1.52	9(0)
8	Oyster	183.7	196.2	8.2	42.3	2.07	9(0)

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

Appendix 32. Cefas laboratory mean and assigned mean concentrations (µg/kg), and within (RSD_r) and between (RSD_R) laboratory relative standard deviations, and HorRat values for the determination of AZA toxins obtained in the RIKILT LC-MS/MS collaborative study (2010).

Sample	Matrix	Lab. mean (µg/kg)	Assigned mean (µg/kg)	RSD_r (%)	RSD_R (%)	HorRat value	Nos. of Labs a(b)
AZA1 (as determined from AZA1 calibration; r^2 = 1.00; response drift = 9.6%)							
1	Mussel	68.7	71.0	11.2	17.3	0.73	11(0)
2	Mussel	<LOQ	<LOQ				
3	Mussel	110.1	115.5	2.8	9.1	0.41	11(0)
4	Oyster	179.1	194.5	2.9	12.2	0.6	11(0)
5	Mussel	<LOQ	<LOQ				
6	Mussel	250.8	252.2	3.3	10.2	0.52	10(0)
7	Cockle	144.9	142.4	2.7	11.4	0.53	10(0)
8	Oyster	<LOQ	<LOQ				
AZA2 (as determined from AZA2 calibration; r^2 = 1.00; response drift = -1.2%)							
1	Mussel	16.6	17.2	10.6	20	0.68	12(0)
2	Mussel	<LOQ	<LOQ				
3	Mussel	27.7	27.5	3	9.5	0.35	12(0)
4	Oyster	42.0	44.6	5.6	8.8	0.34	12(0)
5	Mussel	<LOQ	<LOQ				
6	Mussel	59.3	59.8	8.3	9.1	0.37	12(0)
7	Cockle	33.4	35.5	5.7	7.2	0.27	11(1)
8	Oyster	<LOQ	<LOQ				
AZA2 (as determined from AZA1 calibration; r^2 = 1.00; response drift = 9.6%)							
1	Mussel	17.1	16.0	3.7	11.2	0.37	9(2)
2	Mussel	<LOQ	<LOQ				
3	Mussel	28.0	24.8	2.9	10.3	0.37	10(1)
4	Oyster	42.1	40.4	2.7	12.3	0.48	10(1)
5	Mussel	<LOQ	<LOQ				
6	Mussel	59.2	56.6	3.3	14.4	0.58	10(0)
7	Cockle	33.7	32.1	3.7	13	0.48	10(0)
8	Oyster	<LOQ	<LOQ				
AZA3 (as determined from AZA3 calibration; r^2 = 1.00; response drift = -1.5%)							
1	Mussel	48.9	53.5	3.7	6.3	0.25	11(1)
2	Mussel	3.9	<LOQ				
3	Mussel	82.1	84.4	3.4	5.7	0.25	11(1)
4	Oyster	76.7	81.4	3.9	6.5	0.28	11(1)
5	Mussel	<LOQ	<LOQ				
6	Mussel	188.5	186	1.9	10.9	0.49	11(1)
7	Cockle	102.5	108.9	3.5	7.1	0.32	11(1)
8	Oyster	<LOQ	<LOQ				
AZA3 (as determined from AZA1 calibration; r^2 = 1.00; response drift = 9.6%)							
1	Mussel	33.4	37.3	5.0	19.7	0.75	11(0)
2	Mussel	4.6	<LOQ				
3	Mussel	54.6	55.9	2.2	18.4	0.74	10(1)
4	Oyster	51.2	54.5	3.7	21	0.85	10(1)
5	Mussel	<LOQ	<LOQ				
6	Mussel	122.7	126.7	2.1	15.4	0.71	9(1)
7	Cockle	67.7	72.6	3.3	17	0.72	9(1)
8	Oyster	<LOQ	<LOQ				

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

Appendix 33. Cefas laboratory mean and assigned mean concentrations (µg/kg), and within (RSD_r) and between (RSD_R) laboratory relative standard deviations (RSD) and HorRat values for the determination of PTX2 and YTX toxins obtained in the RIKILT LC-MS/MS collaborative study.

Sample	Matrix	Lab. mean (µg/kg)	Assigned mean (µg/kg)	RSD_r (%)	RSD_R (%)	HorRat value	Nos. of Labs a(b)
PTX2 (as determined from PTX2 calibration; r^2 = 1.00; response drift = 0.4%)							
1	Mussel	<LOQ	<LOQ				
2	Mussel	85.5	82.3	na	10.2	0.44	10(0)
3	Mussel	<LOQ	<LOQ				
4	Oyster	<LOQ	<LOQ				
5	Mussel	11.1	9.7	10.9	34.0	1.06	11(0)
6	Mussel	<LOQ	<LOQ				
7	Cockle	14.7	15.2	7.4	9.7	0.32	9(2)
8	Oyster	180.5	186.3	4.3	20.6	1.00	11(0)
YTX (as determined from YTX calibration; r^2 = 0.98; response drift = 2.9%)							
1	Mussel	116	143.9	12.5	12.5	0.58	9(1)
2	Mussel	1978	1023.2	5.4	12.8	0.81	9(1)
3	Mussel	946	713.2	6.3	8.3	0.49	9(1)
4	Oyster	<LOQ	<LOQ				
5	Mussel	311	290.6	11.1	16.8	0.87	11(0)
6	Mussel	147	206.9	12.3	13.5	0.66	10(1)
7	Cockle	168	172.7	12.4	9.9	0.48	9(2)
8	Oyster	<LOQ	39.1	6.2	48.4	1.86	11(0)
45 OH YTX (as determined from YTX calibration; r^2 = 0.98; response drift = 2.9%)							
1	Mussel	98.8	98.6	8	32.5	1.43	10(0)
2	Mussel	1172.9	685.9	3	40	2.35	10(0)
3	Mussel	792.2*	418.2	6.5	27.3	1.49	9(1)
4	Oyster	<LOQ	<LOQ				
5	Mussel	264.3	177.7	13.1	33.9	1.63	11(0)
6	Mussel	161.9	114.9	15.5	37.3	1.68	11(0)
7	Cockle	121.4	108.6	16.9	40.5	1.81	11(0)
8	Oyster	<LOQ	<LOQ				

a= number of labs remaining after removal of number of outliers indicated by (b).
Sample 2 (PTX2 spiked at 95.7 µg/kg)
Sample 8 (PTX2 spiked at 202.5 µg/kg)

Appendix 34. Cefas laboratory mean and assigned mean concentrations (µg/kg), and within (RSD_r) and between (RSD_R) laboratory relative standard deviations (RSD) and HorRat values for the determination of total OA obtained in the EU-RL collaborative study.

Sample Nos.	Shellfish Matrix	Toxin calibration (solution)	Concentration correction	Lab mean	Assigned mean	RSD[r]	RSD[R]	HorRat Value	Nos. of Labs a(b)
1&3	Wedge shell clam	OA (individual)	uncorrected	152	184	10.8	28.6	1.39	15(0)
		OA (multi-toxin)	uncorrected	157	177	10.5	28.9	1.39	15(0)
		OA (individual)	MMS	156	143	11.1	24.4	1.14	15(0)
		OA (multi-toxin)	MMS	151	141	11.2	24.5	1.14	15(0)
		OA (individual)	Mus-b CRM	186	175	10.8	26.1	1.26	14(0)
		OA (multi-toxin)	Mus-b CRM	190	175	10.7	27.4	1.32	14(0)
2&5	Razor clam	OA (individual)	uncorrected	70.6	84.6	12.4	32.7	1.41	12(3)
		OA (multi-toxin)	uncorrected	82.3	82.1	12.3	36.9	1.58	12(3)
		OA (individual)	MMS	72.5	63.3	18.8	25.2	1.04	12(3)
		OA (multi-toxin)	MMS	79.2	64.8	17.4	26.9	1.11	12(3)
		OA (individual)	Mus-b CRM	86.3	83.6	19.0	37.3	1.61	12(2)
		OA (multi-toxin)	Mus-b CRM	100	87.9	16.7	39.8	1.73	12(2)
4&6	Mussel (uncooked)	OA (individual)	uncorrected	70.6	86.0	12.5	31.2	1.35	13(1)
		OA (multi-toxin)	uncorrected	82.3	74.2	10.5	20.7	0.88	11(4)
		OA (individual)	MMS	72.5	65.5	11.5	18.3	0.76	13(1)
		OA (multi-toxin)	MMS	79.1	66.6	10.6	21.5	0.89	13(2)
		OA (individual)	Mus-b CRM	86.3	80.0	12.7	31.3	1.34	12(1)
		OA (multi-toxin)	Mus-b CRM	100	84.1	11.3	36.7	1.58	12(2)
7&8	Striped Venus clam	OA (individual)	uncorrected	150	182	8.34	34.1	1.65	14(1)
		OA (multi-toxin)	uncorrected	133	174	7.9	35.3	1.7	14(1)
		OA (individual)	MMS	156	154	12.9	35.1	1.66	15(0)
		OA (multi-toxin)	MMS	157	158	9.57	36.3	1.72	14(1)
		OA (individual)	Mus-b CRM	188	175	9.87	16.3	0.79	12(2)
		OA (multi-toxin)	Mus-b CRM	188	175	9.69	17.7	0.85	12(2)
9&10	Mussel (cooked)	OA (individual)	uncorrected	366	431	4.87	35.5	1.95	15(0)
		OA (multi-toxin)	uncorrected	326	381	5.39	25.5	1.38	14(1)
		OA (individual)	MMS	380	367	5.29	32.0	1.72	13(2)
		OA (multi-toxin)	MMS	385	344	4.82	30.1	1.6	14(1)
		OA (individual)	Mus-b CRM	458	406	5.32	13.1	0.71	12(2)
		OA (multi-toxin)	Mus-b CRM	461	404	5.32	12.7	0.69	12(2)
11&14	Cockle	OA (individual)	uncorrected	109	154	22.2	39.7	1.87	15(0)
		OA (multi-toxin)	uncorrected	99.5	128	13.1	34.7	1.59	13(2)
		OA (individual)	MMS	91.4	115	15.3	36.0	1.63	13(1)
		OA (multi-toxin)	MMS	91.3	113	16.4	34.7	1.56	13(1)
		OA (individual)	Mus-b CRM	117	127	12.2	36.1	1.65	12(2)
		OA (multi-toxin)	Mus-b CRM	116	147	24.2	51.0	2.39	14(0)
12&13	Mussel (cooked)	OA (individual)	uncorrected	277	338	4.38	32.3	1.72	13(2)
		OA (multi-toxin)	uncorrected	254	303	4.13	27.3	1.43	13(2)
		OA (individual)	MMS	233	264	4.62	31.9	1.63	12(2)
		OA (multi-toxin)	MMS	233	253	4.39	27.5	1.4	12(2)
		OA (individual)	Mus-b CRM	297	311	4.07	31.4	1.65	12(2)
		OA (multi-toxin)	Mus-b CRM	297	306	3.99	31.3	1.64	12(2)

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

Appendix 35. Cefas laboratory mean and assigned mean concentrations (µg/kg), and within (RSD_r) and between (RSD_R) laboratory relative standard deviations (RSD) and HorRat values for the determination of total DTX1 obtained in the EU-RL collaborative study.

Sample Numbers	Shellfish Matrix	Toxin calibration (solution)	Concentration correction	Lab mean	Assigned mean	RSD[r]	RSD[R]	HorRat Value	Nos. of Labs a(b)
1&3	Wedge shell clam	OA (individual)	uncorrected						
		DTX1 (multi-toxin)	uncorrected						
		OA (individual)	MMS						
		DTX1 (multi-toxin)	MMS						
		OA (individual)	Mus-b CRM						
		DTX1 (multi-toxin)	Mus-b CRM						
2&5	Razor clam	OA (individual)	uncorrected						
		DTX1 (multi-toxin)	uncorrected						
		OA (individual)	MMS						
		DTX1 (multi-toxin)	MMS						
		OA (individual)	Mus-b CRM						
		DTX1 (multi-toxin)	Mus-b CRM						
4&6	Mussel (uncooked)	OA (individual)	uncorrected	330	422	5.84	31	1.7	14(1)
		DTX1 (multi-toxin)	uncorrected	275	388	8.48	27.2	1.48	15(0)
		OA (individual)	MMS	251	249	9.36	20.5	1.04	14(1)
		DTX1 (multi-toxin)	MMS	241	251	6.33	21.9	1.11	13(2)
		OA (individual)	Mus-b CRM	340	312	8.86	16.4	0.86	13(1)
		DTX1 (multi-toxin)	Mus-b CRM	345	301	8.36	18.1	0.95	14(1)
7&8	Striped Venus clam	OA (individual)	uncorrected						
		DTX1 (multi-toxin)	uncorrected						
		OA (individual)	MMS						
		DTX1 (multi-toxin)	MMS						
		OA (individual)	Mus-b CRM						
		DTX1 (multi-toxin)	Mus-b CRM						
9&10	Mussel (cooked)	OA (individual)	uncorrected						
		DTX1 (multi-toxin)	uncorrected						
		OA (individual)	MMS						
		DTX1 (multi-toxin)	MMS						
		OA (individual)	Mus-b CRM						
		DTX1 (multi-toxin)	Mus-b CRM						
11&14	Cockle	OA (individual)	uncorrected						
		DTX1 (multi-toxin)	uncorrected						
		OA (individual)	MMS						
		DTX1 (multi-toxin)	MMS						
		OA (individual)	Mus-b CRM						
		DTX1 (multi-toxin)	Mus-b CRM						
12&13	Mussel (cooked)	OA (individual)	uncorrected	188.6	273	6.66	39.2	2.01	15(0)
		DTX1 (multi-toxin)	uncorrected	163.8	244	6.4	37.1	1.87	15(0)
		OA (individual)	MMS	136.8	142	7.29	16.1	0.75	12(2)
		DTX1 (multi-toxin)	MMS	136.8	251	6.33	21.9	1.11	13(2)
		OA (individual)	Mus-b CRM	176.7	205	6.53	21.5	1.06	12(2)
		DTX1 (multi-toxin)	Mus-b CRM	178.5	207	6.67	35.2	1.74	14(0)

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

Appendix 36. Cefas laboratory mean and assigned mean concentrations (µg/kg), and within (RSD_r) and between (RSD_R) laboratory relative standard deviations (RSD) and HorRat values for the determination of total DTX2 obtained in the EU-RL collaborative study.

Sample Numbers	Shellfish Matrix	Toxin calibration (solution)	Concentration correction	Lab mean	Assigned mean	RSD[r]	RSD[R]	HorRat Value	Nos. of Labs a(b)
1&3	Wedge shell clam	OA (individual)	uncorrected	187	238	8.80	27.4	1.38	14(1)
		DTX2 (multi-toxin)	uncorrected	162	191	8.28	20.3	0.99	13(2)
		OA (individual)	MMS	159	171	12.6	32.9	1.58	15(0)
		DTX2 (multi-toxin)	MMS	152	172	13.8	37.1	1.78	15(0)
		OA (individual)	Mus-b CRM	228	228	8.44	25.7	1.29	13(1)
		DTX2 (multi-toxin)	Mus-b CRM	197	201	7.93	25.0	1.23	13(1)
2&5	Razor clam	OA (individual)	uncorrected	57.3	68.2	14.9	23.2	0.97	11(2)
		DTX2 (multi-toxin)	uncorrected	59.7	65.1	12.7	34.1	1.41	13(1)
		OA (individual)	MMS	48.9	51.1	11.8	43	1.72	12(1)
		DTX2 (multi-toxin)	MMS	56.1	53.3	11.3	36.8	1.48	13(1)
		OA (individual)	Mus-b CRM	70.1	69.8	13.8	33.2	1.39	12(1)
		DTX2 (multi-toxin)	Mus-b CRM	72.7	64.4	12.6	36.4	1.51	13(1)
4&6	Mussel (uncooked)	OA (individual)	uncorrected						
		DTX2 (multi-toxin)	uncorrected						
		OA (individual)	MMS						
		DTX2 (multi-toxin)	MMS						
		OA (individual)	Mus-b CRM						
		DTX2 (multi-toxin)	Mus-b CRM						
7&8	Striped Venus clam	OA (individual)	uncorrected	70.5	80.4	19.7	34.4	1.47	14(0)
		DTX2 (multi-toxin)	uncorrected	56.3	72.2	19.1	35.1	1.48	13(0)
		OA (individual)	MMS	51.8	59.6	22.4	34.4	1.41	14(0)
		DTX2 (multi-toxin)	MMS	50.8	57.5	22.2	40.1	1.63	14(0)
		OA (individual)	Mus-b CRM	88.3	73.2	10.8	26.1	1.1	12(2)
		DTX2 (multi-toxin)	Mus-b CRM	79.6	71.6	20.5	35.8	1.5	13(0)
9&10	Mussel (cooked)	OA (individual)	uncorrected	46.5	57.7	8.03	34.5	1.4	11(1)
		DTX2 (multi-toxin)	uncorrected	36.4	46.1	8.78	39.2	1.54	12(1)
		OA (individual)	MMS	34.2	37.3	8.76	32.6	1.24	11(1)
		DTX2 (multi-toxin)	MMS	32.9	37.5	9.4	40.4	1.54	12(1)
		OA (individual)	Mus-b CRM	58.3	52.7	8.25	32.6	1.31	11(1)
		DTX2 (multi-toxin)	Mus-b CRM	51.5	46.5	8.72	44.1	1.74	12(1)
11&14	Cockle	OA (individual)	uncorrected	75.1	104	21.9	33.1	1.47	15(0)
		DTX2 (multi-toxin)	uncorrected	55.9	90.1	23.6	41.3	1.80	14(0)
		OA (individual)	MMS	58.9	63.1	11.9	33.1	1.37	12(2)
		DTX2 (multi-toxin)	MMS	55.7	63.7	11.6	33.8	1.40	12(2)
		OA (individual)	Mus-b CRM	80.6	98.2	24.1	40.7	1.80	14(0)
		DTX2 (multi-toxin)	Mus-b CRM	65.5	89.7	24.1	45.9	2.00	13(0)
12&13	Mussel (cooked)	OA (individual)	uncorrected	303	400	6.32	32.3	1.76	14(1)
		DTX2 (multi-toxin)	uncorrected	248	352	7.38	34.5	1.85	15(0)
		OA (individual)	MMS	238	247	6.89	22.4	1.13	13(1)
		DTX2 (multi-toxin)	MMS	247	242	6.91	20.5	1.04	13(1)
		OA (individual)	Mus-b CRM	325	350	5.73	27.6	1.47	12(2)
		DTX2 (multi-toxin)	Mus-b CRM	290	356	7.27	38.4	2.06	14(0)

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

Appendix 37. Cefas laboratory mean and assigned mean concentrations (µg/kg), and within (RSD_r) and between (RSR_R) laboratory relative standard deviations (RSD) and HorRat values for the determination of PTX2 obtained in the EU-RL collaborative study.

Sample Numbers	Shellfish Matrix	Toxin calibration (solution)	Concentration correction	Lab mean	Assigned mean	RSD[r]	RSD[R]	HorRat Value	Nos. of Labs a(b)
1&3	Wedge shell clam	PTX2 (individual)	uncorrected	80.3	82.9	12.6	32.8	1.41	13(0)
		PTX2 (multi-toxin)	uncorrected	104	87.0	8.53	24.9	1.08	12(0)
		PTX2 (individual)	MMS	72.3	90.4	10.5	38.6	1.68	13(0)
		PTX2 (multi-toxin)	MMS	81.1	96.4	10.1	30.7	1.35	12(0)
2&5	Razor clam	PTX2 (individual)	uncorrected						
		PTX2 (multi-toxin)	uncorrected						
		PTX2 (individual)	MMS						
		PTX2 (multi-toxin)	MMS						
4&6	Mussel (uncooked)	PTX2 (individual)	uncorrected						
		PTX2 (multi-toxin)	uncorrected						
		PTX2 (individual)	MMS						
		PTX2 (multi-toxin)	MMS						
7&8	Striped Venus clam	PTX2 (individual)	uncorrected	93.0	77.7	9.86	34.0	1.45	13(0)
		PTX2 (multi-toxin)	uncorrected	110	98.9	12.1	26.1	1.15	12(0)
		PTX2 (individual)	MMS	81.9	76.7	8.52	28.6	1.21	12(1)
		PTX2 (multi-toxin)	MMS	85.0	81.1	6.70	18.5	0.79	11(1)
9&10	Mussel (cooked)	PTX2 (individual)	uncorrected						
		PTX2 (multi-toxin)	uncorrected						
		PTX2 (individual)	MMS						
		PTX2 (multi-toxin)	MMS						
11&14	Cockle	PTX2 (individual)	uncorrected						
		PTX2 (multi-toxin)	uncorrected						
		PTX2 (individual)	MMS						
		PTX2 (multi-toxin)	MMS						
12&13	Mussel (cooked)	PTX2 (individual)	uncorrected						
		PTX2 (multi-toxin)	uncorrected						
		PTX2 (individual)	MMS						
		PTX2 (multi-toxin)	MMS						

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

Appendix 38. Cefas laboratory mean and assigned mean concentrations (µg/kg), and within (RSD_r) and between (RSR_R) laboratory relative standard deviations (RSD) and HorRat values for the determination of AZA1 obtained in the EU-RL collaborative study.

Sample Numbers	Shellfish Matrix	Toxin calibration (solution)	Concentration correction	Lab mean	Assigned mean	RSD[r]	RSD[R]	HorRat Value	Nos. of Labs a(b)
1&3	Wedge shell clam	AZA1 (individual)	uncorrected	88.9	80.4	11.5	16.9	0.72	13(1)
		AZA1 (multi-toxin)	uncorrected	83.9	82.3	10.90	14.2	0.61	13(1)
		AZA1 (individual)	MMS	78.5	91.5	14.8	28.7	1.25	14(0)
		AZA1 (multi-toxin)	MMS	79.5	85.2	12.4	22.2	0.96	13(1)
2&5	Razor clam	AZA1 (individual)	uncorrected	53.6	47.8	12.0	23.9	0.94	13(0)
		AZA1 (multi-toxin)	uncorrected	49.6	52.2	13.7	26.4	1.06	14(0)
		AZA1 (individual)	MMS	47.3	50.5	12.6	17.8	0.71	13(1)
		AZA1 (multi-toxin)	MMS	47.0	50.6	17.8	17.2	0.69	13(1)
4&6	Mussel (uncooked)	AZA1 (individual)	uncorrected	391	347	10.2	25.8	1.38	13(1)
		AZA1 (multi-toxin)	uncorrected	378	349	9.57	25.4	1.36	13(1)
		AZA1 (individual)	MMS	346	323	6.66	10.7	0.57	10(4)
		AZA1 (multi-toxin)	MMS	358	354	9.99	23	1.25	13(1)
7&8	Striped Venus clam	AZA1 (individual)	uncorrected	127	109	5.14	19.9	0.89	13(2)
		AZA1 (multi-toxin)	uncorrected	122	112	5.11	17.9	0.81	13(2)
		AZA1 (individual)	MMS	121	113	5.79	17.9	0.8	14(1)
		AZA1 (multi-toxin)	MMS	119	111	6.58	20.4	0.92	14(1)
9&10	mussel (cooked)	AZA1 (individual)	uncorrected	261	231	3.85	28.9	1.45	14(1)
		AZA1 (multi-toxin)	uncorrected	248	229	3.75	25.6	1.28	14(1)
		AZA1 (individual)	MMS	250	225	3.99	15.2	0.76	1491)
		AZA1 (multi-toxin)	MMS	241	226	4.73	21.3	1.06	15(0)
11&14	Cockle	AZA1 (individual)	uncorrected	40.9	40.7	6.55	14.5	0.56	12(2)
		AZA1 (multi-toxin)	uncorrected	39.4	42.1	6.38	17	0.66	13(2)
		AZA1 (individual)	MMS	40.9	43.5	11	17.3	0.68	13(1)
		AZA1 (multi-toxin)	MMS	39.9	44.1	11.7	16.6	0.65	13(1)
12&13	Mussel (cooked)	AZA1 (individual)	uncorrected	481	434	5.43	23.1	1.27	14(1)
		AZA1 (multi-toxin)	uncorrected	504	444	5.62	23.5	1.3	14(1)
		AZA1 (individual)	MMS	481	472	3.83	12.8	0.71	12(1)
		AZA1 (multi-toxin)	MMS	510	455	5.42	23.9	1.33	13(1)

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

Appendix 39. Cefas laboratory mean and assigned mean concentrations (µg/kg), and within (RSD_r) and between (RSR_R) laboratory relative standard deviations (RSD) and HorRat values for the determination of AZA2 obtained in the EU-RL collaborative study.

Sample Numbers	Shellfish Matrix	Toxin calibration (solution)	Concentration correction	Lab mean	Assigned mean	RSD[r]	RSD[R]	HorRat Value	Nos. of Labs a(b)
1&3	Wedge shell clam	AZA1 (individual)	uncorrected	30.0	30.3	12.10	20.4	0.76	16(1)
		AZA2 (multi-toxin)	uncorrected	24.8	22.9	12.60	17.7	0.63	12(1)
		AZA1 (individual)	MMS						
		AZA2 (multi-toxin)	MMS	22.3	26.5	16.7	38.3	1.39	13(0)
2&5	Razor clam	AZA1 (individual)	uncorrected	42.9	39.5	6.67	28.6	1.10	11(2)
		AZA2 (multi-toxin)	uncorrected	37.4	36.0	7.39	25.6	0.97	12(1)
		AZA1 (individual)	MMS						
		AZA2 (multi-toxin)	MMS	33.7	39.2	12.9	28.0	1.07	13(0)
4&6	Mussel (uncooked)	AZA1 (individual)	uncorrected	98.3	84.4	10.2	25.1	1.08	12(1)
		AZA2 (multi-toxin)	uncorrected	91.5	74.8	9.22	33.0	1.40	13(1)
		AZA1 (individual)	MMS						
		AZA2 (multi-toxin)	MMS	82.3	75.7	11.0	28.8	1.22	13(1)
7&8	Striped Venus clam	AZA1 (individual)	uncorrected	67.1	63.2	6.91	25.2	1.04	11(3)
		AZA2 (multi-toxin)	uncorrected	64.2	60.2	7.71	12.2	0.50	11(3)
		AZA1 (individual)	MMS						
		AZA2 (multi-toxin)	MMS	54.2	58.8	16.10	23.3	0.95	14(0)
9&10	mussel (cooked)	AZA1 (individual)	uncorrected	71.7	62.1	4.92	28.4	1.17	13(2)
		AZA2 (multi-toxin)	uncorrected	68.6	64.3	4.69	16.4	0.68	12(3)
		AZA1 (individual)	MMS						
		AZA2 (multi-toxin)	MMS	57.9	61.8	5.82	20.1	0.83	15(0)
11&14	Cockle	AZA1 (individual)	uncorrected	39.8	40.0	9.25	24.2	0.93	12(2)
		AZA2 (multi-toxin)	uncorrected	39.6	36.9	7.93	12.2	0.46	11(2)
		AZA1 (individual)	MMS						
		AZA2 (multi-toxin)	MMS	40.5	39.6	11.4	21.0	0.81	12(1)
12&13	Mussel (cooked)	AZA1 (individual)	uncorrected	112	111	1.70	29.2	1.31	11(4)
		AZA2 (multi-toxin)	uncorrected	120	110	5.73	23.7	1.06	13(2)
		AZA1 (individual)	MMS						
		AZA2 (multi-toxin)	MMS	123	109	1.69	23.6	1.06	11(3)

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

Appendix 40. Cefas laboratory mean and assigned mean concentrations (µg/kg), and within (RSD_r) and between (RSR_R) laboratory relative standard deviations (RSD) and HorRat values for the determination of AZA3 obtained in the EU-RL collaborative study.

Sample Numbers	Shellfish Matrix	Toxin calibration (solution)	Concentration Correction	Lab Mean	Assigned Mean	RSD[r]	RSD[R]	HorRat Value	Nos. of Labs a(b)
1&3	Wedge shell clam	AZA1 (individual)	uncorrected						
		AZA3 (multi-toxin)	uncorrected						
		AZA1 (individual)	MMS						
		AZA3 (multi-toxin)	MMS						
2&5	Razor clam	AZA1 (individual)	uncorrected						
		AZA3 (multi-toxin)	uncorrected						
		AZA1 (individual)	MMS						
		AZA3 (multi-toxin)	MMS						
4&6	Mussel (uncooked)	AZA1 (individual)	uncorrected	<LOQ	30.1	12.0	42.6	1.57	8(2)
		AZA3 (multi-toxin)	uncorrected	19.0	22.9	10.3	31.4	1.11	13(1)
		AZA1 (individual)	MMS						
		AZA3 (multi-toxin)	MMS	17.5	21.7	11.2	26.9	0.94	13(1)
7&8	Striped Venus clam	AZA1 (individual)	uncorrected						
		AZA3 (multi-toxin)	uncorrected						
		AZA1 (individual)	MMS						
		AZA3 (multi-toxin)	MMS						
9&10	mussel (cooked)	AZA1 (individual)	uncorrected	82.7	95.3	13.3	25.4	1.12	12(3)
		AZA3 (multi-toxin)	uncorrected	123	102	8.94	30.2	1.34	14(1)
		AZA1 (individual)	MMS						
		AZA3 (multi-toxin)	MMS	118	97.4	9.38	26.3	1.16	15(0)
11&14	Cockle	AZA1 (individual)	uncorrected						
		AZA3 (multi-toxin)	uncorrected						
		AZA1 (individual)	MMS						
		AZA3 (multi-toxin)	MMS						
12&13	Mussel (cooked)	AZA1 (individual)	uncorrected	85	106	7.68	20.4	0.91	12(3)
		AZA3 (multi-toxin)	uncorrected	116	114	7.09	23.5	1.06	14(1)
		AZA1 (individual)	MMS						
		AZA3 (multi-toxin)	MMS	108	105	8.07	19.7	0.88	13(1)

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

Appendix 41. Cefas laboratory mean and assigned mean concentrations (µg/kg), and within (RSD_r) and between (RSR_R) laboratory relative standard deviations (RSD) and HorRat values for the determination of YTX obtained in the EU-RL collaborative study.

Sample Numbers	Shellfish Matrix	Toxin calibration (solution)	Concentration correction	Lab mean	Assigned mean	RSD[r]	RSD[R]	HorRat Value	Nos. of Labs a(b)
1&3	Wedge shell clam	YTX (individual)	uncorrected						
		YTX (multi-toxin)	uncorrected						
		YTX (individual)	MMS						
		YTX (multi-toxin)	MMS						
2&5	Razor clam	YTX (individual)	uncorrected						
		YTX (multi-toxin)	uncorrected						
		YTX (individual)	MMS						
		YTX (multi-toxin)	MMS						
4&6	Mussel (uncooked)	YTX (individual)	uncorrected	133	111	9.16	35.2	1.58	11(0)
		YTX (multi-toxin)	uncorrected	143	105	9.27	27.7	1.23	12(1)
		YTX (individual)	MMS	104	104	9.19	29.3	1.3	11(0)
		YTX (multi-toxin)	MMS	105	101	8.82	29.2	1.29	12(0)
7&8	Striped Venus clam	YTX (individual)	uncorrected						
		YTX (multi-toxin)	uncorrected						
		YTX (individual)	MMS						
		YTX (multi-toxin)	MMS						
9&10	mussel (cooked)	YTX (individual)	uncorrected						
		YTX (multi-toxin)	uncorrected						
		YTX (individual)	MMS						
		YTX (multi-toxin)	MMS						
11&14	Cockle	YTX (individual)	uncorrected						
		YTX (multi-toxin)	uncorrected						
		YTX (individual)	MMS						
		YTX (multi-toxin)	MMS						
12&13	Mussel (cooked)	YTX (individual)	uncorrected						
		YTX (multi-toxin)	uncorrected						
		YTX (individual)	MMS						
		YTX (multi-toxin)	MMS						

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

Appendix 42. Standard operating procedure for the quantitation of marine lipophilic toxins in live bivalve shellfish by liquid chromatography withmass spectrometric detection.

Production Summary

Author:	
Reviewed by:	
Date: March 2011	version 1 Draft
Issue authorisation:	

Distribution of copies

Authorised Recipient	Location

History of Procedure

Issue	Date issued	Changes
Version 1	TBA	

1. AIM AND SCOPE

The aim and scope of the method is to quantify marine lipophilic toxins (MLTs) by liquid chromatography with tandem mass spectrometry (LC-MS/MS) in live bivalve shellfish from the UK statutory biotoxin monitoring programme.

The method must be able to detect and quantify at least the following toxins:

- okadaic acid (OA)
- dinophysistoxins 1 and 2 (DTX1 and DTX2)
- acylated esters of OA, DTX1 and/or DTX2 (*i.e*., DTX3s)
- pectenotoxin 1 and 2 (PTX1 and PTX2)
- azaspiracids 1, 2 and 3 (AZA1, AZA2 and AZA3)
- yessotoxin (YTX)
- 1a-*homo* yessotoxin (*homo* YTX)
- 45 hydroxy yessotoxin (45 OH YTX)
- 45 hydroxy 1a-*homo* yessotoxin (45 OH *homo* YTX).

Implementation of the method for specific shellfish species is dependent upon individual laboratories undertaking the necessary validation work in each species and involving each toxin of relevance. As a minimum, the method must be applicable to the testing of the following species:

- Common mussel
- Common cockle
- Pacific and Native oysters
- King and Queen scallops
- Hard and Razor clams.

Validation must be in accordance with Annex III of Regulation (EC) No 882/2004 and documented in a laboratory validation report. The method must be shown to meet the minimum performance criteria given in Table 1 below when used in-house and be accredited to ISO17025:2005. Laboratories are expected to take part in regular proficiency testing exercises (where available) and to perform satisfactorily in these tests.

Table 1. Validated method performance parameters and minimum performance criteria required to be attained on applying LC-MS/MS to the quantitation of MLTs in live bivalve shellfish.

Performance parameter	**Minimum acceptable performance criteria**
Selectivity	Must demonstrate absence of co-extractive components resulting from impurities, degradant or matrix which may compromise the identity of the analytes
Linearity of detector response	To show detector linearity over working range *i.e*., from limit of quantitation (LOQ) and up to 200% of the regulatory limit. For YTX, the upper LOQ should be at least 150% of the regulatory limit. Linear regression correlation coefficient (r^2) ≥ 0.98 Calibration response drift ≤25% within a batch
Method limit of detection (LOD) [of extraction *plus* analytical methods combined] and dependant of shellfish species	Range of toxin LOD concentrations with s:n ≥3:1 for the quantitation product ion peak: OA 8-16 µg/kg DTX1 9-15 µg/kg DTX2 10-16 µg/kg PTX2 3-4 µg/kg AZA1 1-3 µg/kg YTX 6-46 µg/kg
Method limit of quantitation (LOQ) [of extraction *plus* analytical methods combined] and dependant of shellfish species	Range of toxin LOQ concentrations with s:n ≥10:1 for the quantitation product ion peak: OA 38-58 µg/kg DTX1 23-50 µg/kg DTX2 36-48 µg/kg PTX2 6-10 µg/kg AZA1 4-8 µg/kg YTX 28-105 µg/kg
Recovery range	OA 83-100% [for OA conc. 60 µg/kg] DTX1 89-125% [for DTX1 conc. 100 µg/kg] DTX2 77-103% [for DTX2 conc. 100 µg/kg] PTX2 82-97% [for PTX2 conc. 100 µg/kg] AZA1 80-92% [for AZA1 conc. 100 µg/kg] YTX 74-127% [for YTX conc. 250 µg/kg]
Precision: intra-batch	RSD ≤20% per toxin for each species
Precision: inter-batch	RSD ≤25% per toxin for each species
Measurement uncertainty	Expanded uncertainty of measurement is applied at 95% confidence level to all values determined by LC-MS/MS. The uncertainty associated with the measurement of each toxin analogue, in each sample matrix must be established.
Method ruggedness	Evidence to be shown that extraction and LC-MS/MS methods remain unaffected by small variations in method parameters or where the variation of a parameter is shown to be significant, it is tightly controlled.

The positive identification of the presence of MLTs in shellfish tissues relies on:

a) matching the retention time of analytes in sample extracts with those of the corresponding reference standards and
b) the presence of two multiple reaction monitoring transition ions with the signal-to-noise (s:n) ratio of the confirmatory ion being ≥3.

2. INTRODUCTION

Marine lipophilic toxins (MLTs) are naturally occurring and are produced by certain marine dinoflagellates. Adverse effects such as nausea, vomiting, abdominal cramps, gastrointestinal disorder and diarrhoea may occur following ingestion of shellfish contaminated with certain concentrations of MLTs. Toxins such as okakaic acid, dinophysistoxins and azaspiracids are responsible for the syndrome known as diarrhetic shellfish poisoning (DSP). The European Commission regulations specific to MLT testing in shellfish are Regulation (EC) No. 853/2004 and Regulation (EU) No. 15/2011.

3. PRINCIPLE OF THE METHOD

To isolate MLTs from shellfish tissues, the extraction method is an adaption of the European Union's Reference Laboratory for Marine Biotoxins (EU-RL, 2006) standard operating procedure. A homogenised bivalve molluscan shellfish sample is double extracted with methanol to provide a solvent-to-sample ratio of 10:1 and then filtered prior to LC-MS/MS analysis. To determine esterified forms of okadaic acid and/or dinophysistoxins 1 and/or 2 (*i.e.*, DTX3s), crude methanolic extracts are hydrolysed following Mountford *et al*., (2001) to convert these esters to parent OA/DTX toxins whereby these are quantified by LC-MS/MS to provide total (free *plus* esterified) OA and DTX toxin concentrations.

4. ENVIRONMENTAL CONTROL

Table 2. Storage criteria for whole shellfish, homogenates and extracts prior to official control analysis

Matrix	Conditions to apply upon storage
Whole shellfish	Whole shellfish may be stored for no longer than 72 hours between sample harvest and sample extraction. For example: a) a sample which takes 24 hours to arrive at the laboratory may be stored for a further 48 hours in the laboratory b) a sample which takes 48 hours to arrive may be stored for only 24 hours in the laboratory. Unless the shellfish were already in a frozen state when collected, shellfish should be stored at 2-8^{o}C. Frozen shellfish that have not yet started to thaw may be stored at ≤-20^{o}C.
Shellfish homogenates	Shellfish homogenates prepared for extraction may be stored frozen (≤-20^{o}C) prior to extraction and for no longer than FIVE days
Crude and hydrolysed methanolic extracts	These extracts should be analysed by LC-MS/MS soon after their preparation. However, if this is not possible, extracts may be stored at ≤-20^{o}C for a maximum of FIVE days **in total** from the day of extraction. Care should however be taken to ensure this is clearly marked.

5. SAFETY

Reference should be made to individual laboratory risk assessments and COSHH documentation.

6. CONSUMABLES, EQUIPMENT, CHEMICALS AND REAGENTS

Table 3 list consumables, equipment, reagents and mobile phases required to undertake shellfish tissue extraction and LC-MS/MS analysis. The equipment listed must be within calibration and fit-for-purpose before use.

Table 3. Consumables, equipment, chemicals, reagents & mobile phases.

Type	Item
Equipment/ Apparatus	Gloves and safety glasses 50 mL polypropylene centrifuge tubes with screw caps Homogeniser Vortex mixer Temperature controlled centrifuge 20 mL glass volumetric flasks with glass stoppers Plastic syringes 0.2 µm nylon syringe filters Various calibrated automatic pipettes (5 µL-1000 µL) Autosampler vials and caps Calibrated timer Laboratory film Calibrated pH meter HPLC analytical column (pH compatable) LC and MS/MS instrumentation with electrospray ionisation
Chemicals/ Reagents/ Controls	Water (HPLC grade or better) Methanol (HPLC grade or better) Acetonitrile (HPLC grade or better) 2.5 M sodium hydroxide solution 2.5 M hydrochloric acid solution Ammonium hydrogencarbonate (≥98% purity) Ammonium hydroxide solution
Mobile phases	Mobile phase A: 2 mM ammonium hydrogencarbonate
	Mobile phase B: 90% acetonitrile:2 mM ammonium hydrogencarbonate
Reference standards (see note below)	Including certified reference material (where available) and analytical certified reference standards for at least the following toxins: OA, PTX2, AZA1, YTX. As other toxins become commercially available in certified format, these should be included in the suite of analytes following method characterisation and in-house method validation to establish method performance characteristics as detailed in Table 1.

If certified reference standards are temporarily unavailable, where possible, laboratory reference material (LRM) containing the appropriate toxin analogue(s) must be substituted. Analysis may be undertaken where reference standards or suitable LRM are unavailable, however laboratories, in consultation with the competent authority must consider the impact of the absence of any reference standards in their assessment of the toxicity of the sample.

7. REFERENCE MATERIAL AND PROCEDURAL BLANK

7.1 If certified reference materials are completely or temporarily unavailable, where possible, laboratory reference material (LRM) containing the appropriate range lipophilic toxins must be substituted

7.2. When available, a bulk sample of naturally occurring MLT contaminated material will be used as LRM. Ideally this should contain as many of the regulated toxins as possible. Where this is not possible and as a minimum, at least one toxin representing the OA/DTX, PTX, AZA and YTX groups should be present in the LRM

7.2 The procedural blank is to consist of 2 mL of HPLC grade methanol added to the extraction vessel and taken through the extraction procedure.

8. CONTROL CHECKS AND FREQUENCY

Table 4 lists the controls which must be incorporated within each LC-MS/MS batch.

Table 4. Extraction and analytical method control checks.

Control	Comment	Criteria
Reference material (LRM)		To be extracted and analysed with every batch
Procedural blank		One per batch. Injected directly after an instrumental blank (methanol solvent) following the upper calibration standard
Control checks for standards	Standards	For list of reference standards, see Table 3
Calibration	Number & concentration of reference standards	At least FIVE levels of calibration standard solutions over a concentration range equivalent to at least 10% and ≥200% of the regulatory limits of individual MLTs. For example: OA: 16 to 320 µg/kg PTX2: 16 to 320 µg/kg AZA1: 16 to 320 µg/kg YTX: <0.1 to 2 mg/kg
	Frequency	A set of standards should be run at the beginning and conclusion of the analysis of sample extracts.
Instrumental blank	Water	For LC column equilibration; at least three injections at the beginning of an analytical batch and one at the end of the run.

9. LC and MS PARAMETERS

Table 5 describes typical high performance liquid chromatographic (HPLC) conditions for MLT separation.

Table 5. HPLC separation parameters (after Gerssen *et al*., 2009).

Parameter	Conditions
Run time	Approximately 23 minutes
Injection volume	5-10 µL
Suggested flow rate	0.3 mL/min
Suggested analytical column	C_{18} reversed-phase (150 x 2 mm; 3 µm) pH compatible
Guard Column	Same stationary phase as analytical column
Detection	Tandem mass spectrometric
Column temperature	Dependent on in-house validation conditions
Suggested mobile phase gradient (dependent upon in-house validation)	0-1 min 25% B 1-11 min 100% B 11-17 min 100% B 17-18 min 25% B 18-23 min 25% B

The above conditions may be modified to permit complete chromatographic baseline resolution of okadaic acid (OA) and dinophysistoxin 2 toxins (DTX2).

Mass spectrometric parameters such as capillary and cone voltages, source and desolvation temperatures, gas flows and collision cell energies should be optimised to generate (1) precursor and (2) two product (multiple reaction monitoring) ions for each lipophilic toxin.

10. PREPARATION OF SAMPLES

10.1 Clean the outside of the shellfish with cold running water, if necessary.

10.2 Rinse the inside, only if necessary, with fresh, cold running water and drain.

10.3 Remove the tissue from the shell with a suitable knife or scalpel and place in a sieve to drain and transfer to a blender and blend until homogeneous.

11. PREPARATION OF CRUDE AND HYDROLYSED EXTRACTS

The preparation of crude (unhydrolysed) and hydrolysed extracts are described in the EU-RL (2006) standard operating procedure.

12. LC-MS/MS ANALYSIS

12.1 Proceed with LC-MS/MS analysis in accordance with each test laboratories LC-MS/MS instrumentation procedure.

12.2 Control checks and frequency should be as detailed in Table 4.

13. QUALITY ASSURANCE AND SAMPLE ANALYSIS

13.1 Instrument sensitivity:

The main target peak (quantitation ion) of the LOQ standard must display a signal to noise ratio of ≥ 10 for OA, DTX1, DTX2, PTX2, AZA1 and YTX

13.2 Retention time stability:

The retention time drift of each MLT within the run must be ≤±2.5%.

13.3 Procedural blank:

The chromatogram should ideally be clear of any contamination peak with a signal to noise ratio ≥3 at the toxin retention times. Where a peak is present in the procedural blank and at the predicted retention time of a target toxin and with a signal-to-noise ratio ≥3, the peak area must be subtracted from peak areas present in analysed sample extracts

13.4 LRM:

The calculated toxin concentrations for the peaks of interest (depending on the toxin content of the LRM) must be recorded for each toxin analysed. Clear procedures must be specified for values falling outside of the action and warning limits of control charts.

13.5 Standards:

13.5.1 Peak area responses must be measured for each toxin quantitation peak in the initial calibration of each of the toxin mixes. Correlation coefficients (r^2) must be calculated for the quantitation ion peak of each toxin from a minimum of five different concentration levels and r^2 should be ≥ 0.98.

13.5.2 Where appropriate, a continuing calibration check (CCC) must be run through the sequence after every 20 injections. CCC results are generated by quantifying the

response of the CCC against the initial calibration, enabling the determination of whether the initial calibration is still applicable. CCC results analysed during the sequence must fall within 25% of the expected value.

13.6 In addition, laboratories are expected to take part in regular proficiency testing exercises (where available) and to perform satisfactorily in these tests.

14. DATA ANALYSIS

14.1 Providing the LC-MS/MS batch has been deemed acceptable, each sample chromatogram must be assessed individually for the presence or absence of each MLT. This must include the instrument blank(s), procedural blank, calibration standards, LRM and real samples.

14.2 Carefully check the integration of toxin peaks ensuring the baselines drawn accurately represent the most likely true baseline of the peak. Care should be taken when integrating early or closely-eluting peaks, particularly on sloping baselines.

14.3 Use the peak areas recorded to generate calibrations for each of the toxin analysed, using the results to calculate both correlation coefficients (r^2) and linear calibration equations.

14.4 Record the shellfish species for each sample and use the appropriate values for expanded measurement uncertainty for each sample.

14.5 OA, DTX1, DTX2, PTX1 and PTX2 concentrations are to be expressed as OA equivalence (µg[OA eq.]/kg) after applying toxicity equivalent factors (TEFs) to OA, DTX1, DTX2, PTX1 and PTX2 absolute concentrations. TEFs are detailed in Table 6.

14.6 AZA1, AZA2 and AZA3 concentrations are to be expressed as the sum of AZA1 equivalence (µg[AZA1 eq.]/kg) after applying TEFs to AZA1, AZA2 and AZA3 absolute concentrations.

14.7 YTX, *homo* YTX, 45 OH YTX and 45 OH *homo* YTX concentrations are to be expressed as YTX equivalence (µg[YTX eq.]/kg) after applying toxicity TEFs to YTX, *homo* YTX, 45 OH YTX and 45 OH *homo* YTX absolute concentrations.

14.8 Individual toxin concentrations are only to be included in the total toxicity (*i.e.,* OA eq., AZA1 eq., or YTX eq.) if the value is ≥LOQ for that particular toxin. Three final values per toxin group (*i.e*., OA eq., AZA1 eq., or YTX eq.) are to be reported. These are the lower, actual and higher concentration values based upon the

subtraction, non-application and addition of measurement uncertainty to the individual concentrations after applying TEF values. Concentrations must be calculated using the correct species-specific values for measurement uncertainty estimated during in-house validation of extraction *plus* LC-MS/MS methods.

14.9 Results are reported without correction for toxin recovery.

Table 6. Toxicity equivalent factors for individual lipophilic toxins (EFSA 2008a; 2008b; 2008c; 2009)

Lipophilic toxin	**Abbreviation**	**Toxicity equivalent factors (TEFs)**
Okadaic acid	OA	1.0
Dinophysistoxin 1	DTX1	1.0
Dinophysistoxin 2	DTX2	0.6
Pectenotoxin 1	PTX1	1.0
Pectenotoxin 2	PTX2	1.0
Azaspiracid 1	AZA1	1.0
Azaspiracid 2	AZA2	1.8
Azaspiracid 3	AZA3	1.4
Yessotoxin	YTX	1.0
1a-*homo* yessotoxin	*homo* YTX	1.0
45 OH yessotoxin	45 OH YTX	1.0
45 OH 1a-*homo* yessotoxin	45 OH *homo* YTX	0.5

15. REFERENCES

Commission Regulation (EU) 15/2011 of 10 January 2011 amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalves. *Official Journal of the European Union,* **L6.** 3-6.

European Food Safety Authority. (2008a). Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on marine biotoxins in shellfish – okadaic acid and analogues. *The EFSA Journal*, **589**, 1-62.

European Food Safety Authority. (2008b). Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on Marine biotoxins in shellfish – yessotoxin group. *The EFSA Journal*, **907**: 1-62.

European Food Safety Authority. (2008c). Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on Marine biotoxins in shellfish –azaspiracid group. *The EFSA Journal*, **723**, 1-52

European Food Safety Authority. (2009). Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on marine biotoxins in shellfish - pectenotoxin group. *The EFSA Journal*, **1109**, 1-47.

European Reference Laboratory for marine biotoxins (EU-RL; 2006). Standard operating procedure for the processing and extraction of shellfish for the subsequent detection of diarrhetic shellfish poisons (okadaic acid and dinophysistoxins), and azaspiracids and pectenotoxins in bivalve molluscs. August 2006. pp. 30.

Gerssen, A., Mulder, P.P.J., McElhinney, M.A. and de Boer, J. (2009). Liquid chromatography–tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. *J. Chromatogr. A*, **1216**, 1421–1430.

Institute of Marine Biosciences, National Research Council of Canada toxin reference standard documents including:

- Supplemental Information for each lipophilic/DSP toxin certified reference standard solution
- Certificates of analysis for lipophilic/DSP toxin reference standards

Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. *Official Journal of the European Union,* **L226/22.**

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