Single laboratory validation of a LC-MS/MS method for the determination of regulated marine lipophilic phycotoxins in shellfish typically tested in the United Kingdom

Steven Morris*, Mariusz Huk, Rebecca Kelly, Monika Rapkova, Kathryn Hutchinson, Clothilde Brunet

Centre for Environment, Fisheries and Aquaculture Science, Barrack Road, Weymouth, Dorset. DT4 8UB. United Kingdom
www.cefas.co.uk

Introduction

The lipid-soluble marine phycotoxins - okadaic acid (OA), dinophysistoxins (DTXs), azaspiracids (AZAs), pectenotoxins (PTXs), yessotoxins (YTXs), spirolides (SPXs) and gymnodimine (GYM) form a class of compounds known as lipophilic toxins (LTs). To ensure consumer protection, monitoring of LTs in shellfish is a regulatory requirement. The current EU 'reference' method for detecting LTs is a qualitative, live animal bioassay [1]. Under current EU regulations [2], a series of analytical and/or *in vitro* methods shall be used as 'alternatives' or supplementary to the animal assay. Alternative methods should provide an equivalent level of public health protection and should not be less effective than the assay. Additionally, they should either alone or combined, be able to detect at least the following toxins; OA, DTX1,2, 'DTX3s' (OA/DTX acyl esters) *plus* PTX1,2 as 160 µg[OA equivalents]/kg; AZA1,2,3 (160 µg[AZA equivalents]/kg and YTX, *homo* YTX, 45 OH YTX and 45 OH *homo* YTX (1mg[YTX equivalents]/kg). Animal assays shall also be replaced by alternative detection methods following validation to internationally agreed protocols and, as soon as reference materials become available. Of the alternative techniques, the most promising is based on liquid chromatography with tandem mass spectrometry (LC-MS/MS). It has advantages of selectivity, sensitivity, precision, quantitation, and confirmation of toxin identity.

A single laboratory validation (SLV) scheme was conducted to characterise the performance of adopted extraction and analytical procedures for the measurement of most of the EU-regulated LTs in shellfish typically produced in UK waters. The objectives were to investigate the use of LC-MS/MS as an alternative LT monitoring tool with the future aim of reducing reliance on the use of the assay. Validation criteria as detailed in EU regulation [3] including specificity, sensitivity, recovery, precision and ruggedness were investigated and, the International Union of Pure and Applied Chemistry (IUPAC) guidelines for SLV [4] were followed.

Extraction and analytical methodologies

Shellfish matrices and extraction procedure
Bulk and homogenized tissues of the following shellfish species were collected: Common mussels (*Mytilus edulis*); Common cockles (*Cerastoderma edule*); Pacific oysters (*Crassostrea gigas*); Native oysters (*Ostrea edulis*); King scallops (*Pecten maximus*); Queen scallops (*Aequipecten opercularis*); Hard clams (*Mercenaria mercenaria*); and Razor clams (*Enis* spp.). The extraction procedure described by the Community Reference Laboratory for Marine Biotoxins [5] was adopted. A double methanolic extraction was performed on 2.0 g homogenised tissue. The final solvent-to-sample ratio (SSR) was 10:1.

Liquid chromatography mass spectrometry
An alkaline (pH11) LC gradient [7] was adopted and modified following [6]. An *Agilent 1100* LC and a *Waters Ltd. XBridge* column (150 x 2.0 mm; 3.5 µm; 30°C; 0.3 mL/min) were used. Ammonium hydrogencarbonate (2 mM) was added to mobile phases A (100% H_2O) and B (90% CH_3CN:10% H_2O). The gradient was: 0-1 min 25% B; 1-11.4 min 100% B; 11.4 to 16.7 min 100% B; 16.7-17 min 25% B and 17-22.5 min 25% B. A *Waters Ltd. Quattro Micro* triple quadrupole MS with electrospray ionisation was coupled to the LC. Via toxin infusion into the mobile phase, source parameters and collision energies were optimised for each analyte and for the production of a single precursor ion and two product ions per toxin.

Within one LC run, those toxins which efficiently ionised in negative ionisation mode (OA, DTXs, YTXs) eluted between 7.7 and 9.7 min (Figure 1a). For compounds amenable to positive ionisation (PTXs, AZAs, SPX1 and GYM), these eluted between 9.8 and 13.1 min (Figure 1b). With the exception of YTXs, peak area RSDs ($n = 30$ x 10 µL injections) were <10%; YTXs were up to 20%. RRTs (to OA) were 0.2 – 0.4% and baseline widths of peaks ranged from 0.15 min to 0.6 min (for AZA2).

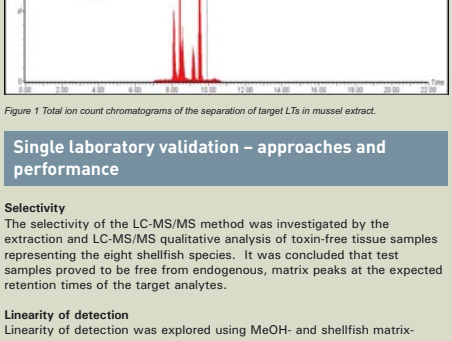


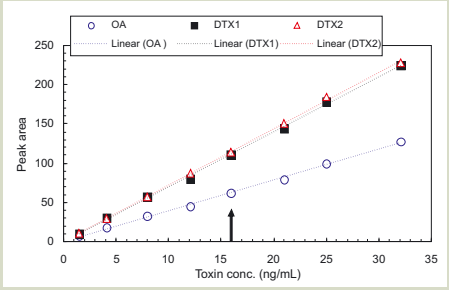
Figure 1 Total ion count chromatograms of the separation of target LTs in mussel extract.

Single laboratory validation – approaches and performance

Selectivity
The selectivity of the LC-MS/MS method was investigated by the extraction and LC-MS/MS qualitative analysis of toxin-free tissue samples representing the eight shellfish species. It was concluded that test samples proved to be free from endogenous, matrix peaks at the expected retention times of the target analytes.

Linearity of detection
Linearity of detection was explored using MeOH- and shellfish matrix-based solutions (SSR 10:1) containing mixtures of LTs. Concentrations ranged from 15 to 320 µg/kg for OA, DTXs, PTXs, AZAs, SPX1 and GYM and from 1 to 20 mg[YTX]/kg. Replicate ($n = 7$) LC injections were performed randomly for six to eight levels of concentrations.

Calibration curves showed correlation coefficients (r^2) of >0.98. Linear regressions with 1/*x* weighting provided best line fits *i.e.*, lower (standard error) values compared to standard errors determined from quadratic regressions. OA prepared in P. oyster and DTX2 in Q. scallop extract matrices approached significance (*t*-test) for a quadratic fit. Signal responses of DTX2, PTX2, AZA1 and YTX prepared in the mussel extract (SSR 10:1) were seen to be suppressed. PTX2 was also suppressed in the presence of P. oyster matrix and AZA1 in N. oyster and K. scallop matrices. DTX1 responses appeared to be enhanced in clam extracts, and YTX was enhanced R. clam and N. oyster matrix extracts. Deviations (bias %) from the nominal concentrations were from 5 to <25%. Respectively, Figure 2 show linear plots for MeOH-based OA/DTX1/2 and AZA1/2/3.



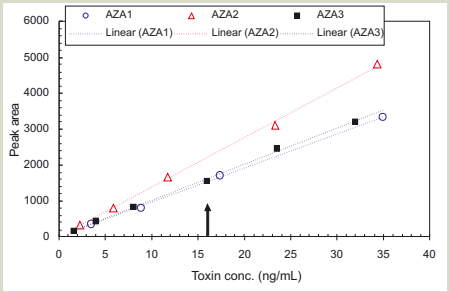


Figure 2. Linear calibration plots for OA/DTX1/2 and AZA1/2/3. Regulatory limits (160[toxin]/kg) are identified by vertical arrows.

Method limits of detection (LOD) and quantitation (LOQ)
LODs and LOQs for the entire (extraction *plus* LC-MS/MS) method were determined experimentally, and by spiking (and extracting) shellfish homogenates ($n = 5$ per species) with low concentrations of analytes. A signal-to-noise ratio criteria of 3:1 (LOD) and 10:1 (LOQ) was applied. Mean method LOD and LOQ concentrations are summarised in Table 1. For OA *and* DTXs, AZA1 *and* PTX2, and YTX, LODs were from 8-16, <5 and <46 µg/kg, respectively. LOQs for the OA/DTX group were between 23 and 58 µg/kg; for AZA1 *and* PTX2 <10 µg/kg, and for YTX from 28-105 µg/kg.

Table 1 Mean method LOD and LOQ values for the determination of selected LTs in representative shellfish

Toxin	Mean method LOD ± 1 s.d. (µg/kg) [RSD %]							
	Common mussel	Common cockle	Pacific oyster	Native oyster	Queen scallop	King scallop	Hard clam	Razor clam
OA	7.77 ± 1.69 [21.7]	11.5 ± 1.85 [16.0]	11.8 ± 0.76 [6.4]	11.8 ± 1.41 [11.9]	8.69 ±2.05 [23.6]	16.0 ± 1.98 [12.4]	11.1 ± 2.43 [21.9]	12.3 ± 3.25 [26.4]
DTX1	14.7 ± 2.0 [13.6]	8.88 ± 1.57 [17.7]	8.73 ± 1.00 [11.5]	11.7 ± 1.60 [13.6]	9.67 ± 1.82 [18.9]	15.3 ± 3.91 [25.5]	10.8 ± 3.08 [28.5]	10.0 ± 2.12 [21.2]
DTX2	10.6 ± 2.0 [18.9]	14.0 ± 2.68 [19.1]	6.76 ± 1.12 [16.6]	14.1 ± 2.63 [18.7]	10.3 ± 1.93 [18.7]	11.8 ± 2.33 [19.7]	8.18 ± 0.97 [11.9]	15.8 ± 2.19 [13.9]
PTX2	2.63 ± 0.14 [5.3]	2.26 ± 0.11 [4.9]	2.39 ± 0.13 [5.4]	3.68 ± 0.19 [5.2]	3.98 ± 0.21 [5.3]	2.85 ± 0.15 [5.3]	3.41 ± 0.17 [5.0]	3.44 ± 0.23 [6.7]
AZA1	2.23 ± 0.16 [7.1]	1.43 ± 0.13 [9.1]	1.10 ± 0.08 [7.2]	3.11 ± 0.08 [2.6]	3.3 ± 0.23 [7.0]	0.62 ± 0.04 [6.5]	2.29 ± 0.12 [4.6]	1.01 ± 0.16 [15.8]
YTX	40.6 ± 1.7 [3.7]	38.3 ± 3.14 [8.2]	35.38 ± 1.08 [20.7]	13.8 ± 1.51 [10.9]	16.2 ± 0.60 [3.7]	17.1 ± 1.01 [5.9]	13.1 ± 0.80 [5.8]	13.9 ± 1.39 [10.0]
SPX1	1.13 ± 0.05 [4.4]	1.03 ± 0.09 [8.7]	0.90 ± 0.06 [6.6]	0.51 ± 0.03 [5.9]	0.44 ± 0.03 [6.8]	0.95 ± 0.06 [6.3]	0.38 ± 0.03 [7.9]	0.34 ± 0.02 [5.9]
GYM	1.44 ± 0.35 [24.3]	1.57 ± 0.24 [15.2]	2.22 ± 0.43 [19.4]	5.29 ± 0.52 [9.9]	2.85 ± 0.52 [18.2]	3.06 ± 0.46 [15.0]	3.48 ± 0.39 [11.2]	3.97 ± 1.10 [27.7]
	Mean method LOQ ± 1 s.d. (µg/kg) [RSD %]							
	Common mussel	Common cockle	Pacific oyster	Native oyster	Queen scallop	King scallop	Hard clam	Razor clam
OA	37.6 ± 7.5 [20.0]	49.6 ± 4.5 [9.1]	48.5 ± 2.69 [5.6]	58.0 ± 5.10 [8.8]	37.7 ± 5.32 [14.1]	43.5 ± 2.52 [5.8]	57.1 ± 10.3 [18.0]	46.7 ± 3.78 [8.1]
DTX1	23.3 ± 3.6 [15.5]	46.4 ± 6.2 [13.4]	41.9 ± 1.85 [4.4]	28.8 ± 3.65 [12.7]	43.4 ± 4.60 [10.6]	30.5 ± 3.67 [12.0]	36.0 ± 6.23 [17.3]	39.0 ± 5.11 [13.1]
DTX2	36.2 ± 5.2 [14.4]	43.0 ± 4.6 [10.7]	43.5 ± 7.36 [16.9]	47.6 ± 1.81 [3.8]	41.6 ± 6.54 [15.7]	38.7 ± 2.71 [7.0]	40.3 ± 2.58 [6.4]	43.8 ± 2.80 [6.4]
PTX2	7.09 ± 0.35 [4.9]	7.56 ± 0.54 [7.1]	6.13 ± 0.10 [1.6]	7.53 ± 0.44 [5.8]	10.1 ± 0.55 [5.5]	6.91 ± 0.28 [4.1]	5.64 ± 0.38 [6.7]	5.95 ± 0.41 [6.9]
AZA1	6.67 ± 0.19 [2.8]	4.30 ± 0.28 [6.5]	3.78 ± 0.28 [7.4]	7.21 ± 0.13 [1.8]	7.65 ± 0.48 [6.3]	3.17 ± 0.05 [1.6]	7.25 ± 0.43 [5.9]	3.23 ± 0.43 [13.3]
YTX	105 ± 16.3 [15.5]	69 0± 13.5 [19.6]	28.2 ± 3.24 [11.5]	48.4 ± 4.02 [8.3]	58.1 ± 4.47 [7.7]	51.3 ± 4.00 [7.8]	71.2 ± 7.48 [10.5]	56.5 ± 5.53 [9.8]
SPX1	2.97 ± 0.15 [5.1]	2.54 ± 0.28 [11.0]	2.53 ± 0.13 [5.1]	2.11 ± 0.08 [3.8]	1.19 ± 0.03 [2.5]	2.59 ± 0.17 [6.6]	1.78 ± 0.15 [8.4]	1.46 ± 0.08 [5.5]
GYM	6.86 ± 0.80 [11.7]	7.42 ± 0.85 [11.5]	12.0 ± 0.94 [7.8]	15.9 ± 2.17 [13.6]	10.8 ± 1.95 [18.4]	11.2 ± 2.85 [25.5]	15.2 ± 3.8 [15.7]	9.28 ± 1.73 [18.6]

Recovery
The efficiency of extraction was assessed by the extraction and analysis of toxin-fortified shellfish tissues. Replicate ($n = 7$) shellfish homogenates were spiked at two levels ('high' and 'low') of concentrations. High level spiking included 60 µg[OA]/kg *plus* 100 µg/kg each of DTX1, DTX2, PTX2, AZA1, SPX1, GYM, and 250 µg[YTX]/kg. Concentrations approximating to method LOQ values were also spiked into samples. One hour was applied post-spiking + mixing, and before extraction. Random LC injections of calibration standard solutions and sample extracts were made within each LC-MS/MS batch. Mean percentage recoveries are presented in Table 2.

Table 2 Mean percentage recoveries ± one standard deviation (s.d.) and relative standard deviations [RSD %] of selected LTs extracted from shellfish matrices fortified with 'high-' and 'low' toxin concentrations

Toxin	Mean percentage recovery ± 1 s.d. [RSD %]							
	Common mussel		Common cockle		Pacific oyster		Native oyster	
	high	low	high	low	high	low	high	low
OA	82.9 ± 9.9 [11.9]	86.9 ± 11.7 [13.4]	95.3 ± 8.1 [8.5]	103 ± 11 [10.5]	86.8 ± 5.1 [5.9]	90.7 ± 8.7 [9.6]	95.1 ± 6.8 [7.2]	118.3 ± 10.7 [9.0]
DTX1	88.6 ± 5.3 [5.9]	70.8 ± 13.7 [19.4]	91.8 ± 8.9 [9.7]	86.0 ± 7.9 [9.1]	91.5 ± 8.5 [9.3]	92.0 ± 7.2 [7.8]	93.4 ± 6.1 [6.5]	95.0 ± 14.3 [15.1]
DTX2	76.9 ± 7.4 [9.6]	81.1 ± 13.6 [16.8]	92.7 ± 5.9 [6.3]	94.5 ± 4.7 [5.0]	89.2 ± 6.0 [6.7]	98.0 ± 9.7 [9.9]	89.7 ± 5.0 [5.6]	99.0 ± 11.4 [11.5]
PTX2	82.2 ± 4.9 [5.9]	136 ± 15 [10.7]	88.1 ± 9.1 [10.3]	95.3 ± 4.7 [4.9]	83.4 ± 3.5 [4.2]	125 ± 11 [7.3]	88.1 ± 2.5 [2.8]	137 ± 6.6 [4.8]
AZA1	80.0 ± 3.8 [4.8]	105 ± 11 [10.0]	88.4 ± 4.2 [4.7]	91.0 ± 2.0 [2.2]	86.1 ± 3.0 [3.5]	98.6 ± 7.2 [7.3]	80.3 ± 3.1 [3.9]	79.7 ± 8.3 [8.0]
YTX	73.7 ± 3.7 [5.0]	85 ± 19 [21.9]	89.3 ± 6.6 [7.4]	89.4 ± 6.4 [7.2]	92.5 ± 10.3 [11.1]	80.5 ± 9.0 [11.2]	105 ± 14.1 [13.4]	103.7 ± 11.2 [10.8]
SPX1	78.8 ± 3.7 [4.7]	70.2 ± 12.5 [17.8]	79.1 ± 5.3 [6.8]	79.7 ± 7.5 [9.4]	76.5 ± 4.2 [5.5]	88.3 ± 8.1 [9.2]	79.9 ± 4.3 [5.4]	70.0 ± 8.0 [11.4]
GYM	82.7 ± 4.2 [5.1]	105 ± 16 [15.2]	92.2 ± 2.8 [3.0]	93.8 ± 4.3 [4.6]	86.4 ± 4.4 [5.0]	96.0 ± 9.1 [9.4]	86.3 ± 3.8 [4.4]	116.7 ± 10.0 [8.6]
	King scallop		Queen scallop		Hard clam		Razor clam	
	high	low	high	low	high	low	high	low
OA	97.4 ± 6.8 [7.0]	94.8 ± 3.4 [3.6]	91.4 ± 11.1 [12.1]	83.0 ± 7 [7.9]	89.1 ± 4.5 [5.1]	65.3 ± 4.3 [6.6]	100 ± 13 [13.0]	97.3 ± 10.7 [10.9]
DTX1	100 ± 5.4 [5.4]	106 ± 6.5 [6.0]	102.4 ± 9.8 [9.6]	97.0 ± 9 [9.4]	110 ± 7.6 [6.9]	82.4 ± 7.9 [9.7]	125 ± 16 [12.6]	133 ± 19 [14.2]
DTX2	94.9 ± 4.2 [4.5]	98.5 ± 4.8 [4.9]	94.5 ± 7.4 [7.8]	98.9 ± 4.1 [4.1]	95.7 ± 4.5 [4.7]	101 ± 9.2 [9.1]	103 ± 8.7 [8.5]	107 ± 7.5 [7.2]
PTX2	94.0 ± 2.1 [2.3]	95.5 ± 2.1 [2.3]	92.4 ± 3.1 [3.4]	113 ± 8.2 [7.2]	93.4 ± 4.4 [4.7]	199 ± 14 [7.0]	97.3 ± 2.9 [3.0]	122 ± 12.6 [10.2]
AZA1	86.7 ± 1.4 [1.6]	85.9 ± 3.4 [3.9]	90.4 ± 2.1 [2.3]	96.7 ± 2.6 [2.7]	92.2 ± 3.3 [3.6]	49.5 ± 2.7 [5.4]	88.7 ± 2.5 [2.9]	94.8 ± 6.6 [7.1]
YTX	88.9 ± 9.3 [9.6]	91.3 ± 6.4 [7.0]	100.2 ± 11.1 [11.0]	131 ± 23 [17.5]	105 ± 8.3 [7.9]	119 ± 15 [12.6]	127 ± 22 [17.3]	67.1 ± 37 [5.6]
SPX1	92.2 ± 4.1 [4.4]	102 ± 9.6 [9.3]	87.9 ± 3.3 [3.7]	115 ± 8.0 [7.0]	87.0 ± 2.6 [2.9]	24.3 ± 4.9 [19.9]	88.7 ± 4.9 [5.6]	56.4 ± 5.1 [8.8]
GYM	96.1 ± 5.1 [5.3]	95.9 ± 5.5 [5.7]	89.5 ± 5.0 [5.6]	99.6 ± 11. [11.1]	87.6 ± 4.9 [5.6]	38.5 ± 4.6 [12.0]	95.3 ± 3.7 [3.9]	100 ± 17 [17.1]

***Intra*-day and *inter*-day precision**
Intra-day repeatability of the entire method was determined from the recovery exercise involving 'high' level fortification, extraction and analysis. Over approximately a two-month period, the *inter*-day precision of the entire method was assessed using three different laboratory operators performing spiking and extraction on different days. Spiking concentrations and sample replication were as above. *Intra*- and *inter*-day precision of the entire method was expressed as the RSD of the mean concentration of each toxin recovered in relation to the shellfish matrix.

Precision values are presented in Table 3. For *intra*-day precision, RSDs were <13% with the exception of YTX determined from oysters/scallops and clams (range: 11.0–17.9%). With the exception of YTX quantified in all shellfish matrices, *inter*-day precision values were <12%. RSDs for YTX measurements were between 11.9% (Q. scallop) and 21.2% (cockle).

Table 3 Intra- and inter-day precision values (RSDs) of LC-MS/MS determinations of selected lipophilic toxins from fortified and extracted shellfish homogenates.

Toxin	Common mussel		Common cockle	
	Intra-day	*Inter-day*	*Intra-day*	*Inter-day*
OA	11.9	9.6	8.5	11.1
DTX1	5.9	5.0	9.7	8.8
DTX2	9.7	8.9	6.3	6.5
PTX2	5.9	9.2	10.3	6.4
AZA1	4.7	4.0	4.7	6.7
YTX	5.0	15.4	7.4	21.2
SPX1	4.7	7.7	6.7	5.1
GYM	5.1	3.1	3.0	5.7
	Pacific oyster		**Native oyster**	
	Intra-day	*Inter-day*	*Intra-day*	*Inter-day*
OA	5.9	9.4	7.2	7.3
DTX1	9.3	6.9	6.4	4.9
DTX2	6.7	8.0	5.6	7.4
PTX2	4.2	4.3	2.8	4.0
AZA1	3.4	4.9	3.9	4.5
YTX	11.1	13.7	13.5	12.7
SPX1	5.5	5.3	5.3	3.6
GYM	5.0	3.8	4.4	4.2
	Queen scallop		**King scallop**	
	Intra-day	*Inter-day*	*Intra-day*	*Inter-day*
OA	12.1	6.9	7.0	8.1
DTX1	9.6	4.9	5.4	6.5
DTX2	7.8	7.0	4.5	4.9
PTX2	3.4	4.0	2.3	3.7
AZA1	2.3	4.7	1.6	3.2
YTX	11.0	11.9	17.2	21.1
SPX1	3.7	6.8	4.4	3.1
GYM	5.6	6.2	5.3	5.2
	Hard clam		**Razor clam**	
	Intra-day	*Inter-day*	*Intra-day*	*Inter-day*
OA	7.3	6.4	13.0	7.1
DTX1	4.9	4.2	12.6	6.2
DTX2	7.3	4.8	8.5	6.6
PTX2	4.0	8.8	3.0	7.8
AZA1	4.4	10.7	3.0	4.3
YTX	12.7	16.5	17.9	8.6
SPX1	3.7	8.4	5.6	5.3
GYM	4.2	4.4	3.9	4.0

Ruggedness
Ruggedness was evaluated following a Plackett-Burman [8] design, using replicated ($n = 3$) shellfish homogenates fortified with 100 µg/kg of OA, DTX1, DTX2, PTX2, SPX1, GYM, 35 µg[AZA1]/kg, and 250 µg[YTX]/kg. Varying one methodological parameter (below and above its nominal value) at a time, five and two minor changes to the extraction and the LC methodologies were introduced. Concentrations of LTs were compared using analysis of variance. Changes to the methods included: MeOH extraction volume (A/a), vortex mixing time (B/b), centrifugation speed (C/c), homogenisation speed (D/d), centrifugation speed (E/e), LC flow rate (F/f) and mobile phase pH (G/g).

- None of the LTs isolated from any of the spiked eight shellfish matrices were significantly influenced ($p > 0.001$) by small changes made to the extraction procedure,
- for co-eluting analytes - PTX2 and SPX1, significantly different ($p < 0.001$) and elevated concentrations in mussel and oyster extracts were quantified after decreasing as well as increasing LC flow rates,
- in all YTX/matrix combinations, a flow rate of 0.29 mL/min resulted in lower, observed YTX concentrations,
- pH changes (pH10.8 and pH11.2) made to the mobile phases appeared to decrease the measured concentrations of PTX2 in mussel, cockle, scallop and clam extracts; pH11.2 resulted in higher YTX concentrations compared to levels determined using a pH10.8 gradient.

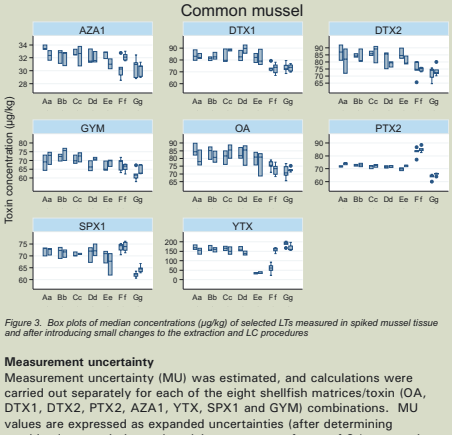


Figure 3. Box plots of median concentrations (µg/kg) of selected LTs measured in spiked mussel tissue and after introducing small changes to the extraction and LC procedures

Measurement uncertainty
Measurement uncertainty (MU) was estimated, and calculations were carried out separately for each of the eight shellfish matrices/toxin (OA, DTX1, DTX2, PTX2, AZA1, YTX, SPX1 and GYM) combinations. MU values are expressed as expanded uncertainties (after determining combined uncertainties and applying a coverage factor of 2 (representing a 95% confidence level).

Combined MUs were first calculated as the square root of the sum of the squares of the standard uncertainties. They comprised precision (RSD) data from validation exercises including: LOD; LOQ; intra-day variation; recovery efficiency, and by including an estimated, standard uncertainty value of 0.2 for reproducibility. Currently, expanded uncertainties are only indicative and were found to be in the range of 0.42 to 0.59. Values are likely to become more robust as additional precision data are made available in the near future

Future work

The efficiency of the entire method will continue to be checked by future participation in proficiency tests organised by QUASIMEME [9]. Our recent results obtained from such exercises and involving the determination of OA/DTX toxins have been satisfactory.

By systematic experimentation, the influence of matrix effects will be studied further using solid phase extraction procedures to: (1) establish the degree of enhancement or suppression effects on LT quantification resulting from the extraction of representative shellfish matrices and; (2) to assess its inclusion in the sequence of applied methods.

A second validation phase will take place in the near future whereby the entire method will be applied intensively to real-time, shellfish samples acquired from national monitoring programmes. Conducted in tandem with animal assays, the extraction and the LC-MS/MS methods will be assessed for their fitness-for-purpose.

Acknowledgements
The authors are grateful to the United Kingdom's Food Standards Agency for funding this programme (FO1R0002/002). The authors also wish to thank Dr. Mike Quilliam and staff at the Institute for Marine Biosciences (National Research Council Canada) for the provision of reference materials used in this validation scheme.

References

[1] Anon. (2004). Regulation (EC) No 853/2004. Official Journal of the European Union L139. Section VII, Chapter V. pp. 60-61.
[2] Anon. (2004). Regulation (EC) No 2074/2005. Official Journal of the European Union 338. Annex III, Chapter III. pp. 40-41.
[3] Anon (1986). Council Directive 86/609/EEC. Official Journal of the European Union L358. Articles 7 and 23.
[4] Anon. (2004). Regulation (EC) No 882/2004. Official Journal of the European Union L 165 Annex III. p. 120
[5] Thompson, M., Ellison, S.L.R. & Wood, R. (2002). Pure and Applied Chemistry, 74, 835-855.
[6] Community Reference Laboratory for Marine Biotoxins. (2006). Multi-toxin reference method for LC-MS analysis for OA, AZA, YTX, PTX and SPX biotoxin groups. Standard operating procedure. pp. 10.
[7] Gerssen, A., Mulder, P.P.J., McElhinney, M.A. & de Boer, J. (2009). J. Chromatogr. A, 1216, 1421–1430.
[8] Plackett, R.L. & Burman, J.P. (1946). Biometrika. 33, 305-325.
[9] http://www.quasimeme.org