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Rapid determination of polyether marine toxins using liquid chromatography–multiple tandem mass spectrometry

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Abstract

The diarrhetic shellfish poisoning (DSP) toxins, okadaic acid (OA), dinophysistoxins (DTX); pectenotoxin-2 (PTX2) and pectenotoxin-2 seco acids, were determined in marine phytoplankton, *Dinophysis acuta*, and mussels (*Mytilus edulis*) collected along the southwest coast of Ireland. Liquid chromatography–multiple tandem mass spectrometry (LC–MS/MS) was employed for the simultaneous determination of a series of marine toxins with large polarity differences. Separation of five DSP toxins was achieved on a C_{18} column (Luna-2, 150 mm × 2.1 mm, 5 μm) using an acetonitrile–water gradient with ammonium acetate as an eluent modifier. Electrospray ionisation (ESI) in negative mode, was used to generate the molecule related ion, $[M-H]^-$, for each toxin. To develop a multiple reaction monitoring (MRM) method, fragmentation studies were performed to determine the optimum precursor-product ion combinations: OA (803/255), DTX2 (803/255), DTX1 (817/255), PTX2SAs (875/137) and PTX2 (857/137). This highly sensitive method had detection limits better than 1 pg (on-column). Linear calibrations were obtained for shellfish extracts that were spiked with toxins, OA, 0.007–1.00 μg/ml (r^2 = 0.9993, N = 3) and DTX2, 0.054–8.5 μg/ml (r^2 = 0.9992, N = 3). Good reproducibility data were also achieved with %RSD values (N = 3) ranging from 3.15% (0.56 μg DTX2/ml) to 5.71% (0.14 μg DTX2/ml), for shellfish extracts. The method was sufficiently sensitive to permit the determination of DSP toxins in small numbers of picked phytoplankton cells (N = 12–40). In one sample of *D. acuta* the average toxin composition per cell was: OA (7.0 pg), DTX2 (11 pg) and PTX2 (7.2 pg).

Keywords: Phytoplankton; Okadaic acid; Dinophysistoxins; Pectenotoxins

1. Introduction

Polyether toxins that contaminate bivalve shellfish, including scallops, mussels, clams and oysters, can cause acute human intoxications. In Europe, these toxins are responsible for the syndromes, diarrhetic shellfish poisoning (DSP) [1] and azaspiracid poisoning (AZP) [2]. Three classes of DSP toxins were initially designated: (a) okadaic acid (OA) and dinophysistoxins (DTXs) [3,4]; (b) pectenotoxins (PTXs) [5,6]; and (c) yessotoxins (YTXs) [7]. Studies in Ireland, Spain and Portugal, have shown that OA and DTX2 (Fig. 1) were the predominant DSP toxins in mussels [8–10]. Strict regulations have been implemented in most EU countries where a limit of 0.16 μg/g for these toxins in shellfish has been established [11]. However, YTXs are no longer classified as DSP toxins as they are non-diarrhetic [11,12].

Most previous studies of DSP toxin profiles employed methods that targeted acidic polyether toxins. One reason for this was that the detection of acidic toxins was dependent on derivatisation with fluorimetric reagents, followed by quantitation using liquid chromatography (LC-FLD). Derivatisation reagents included, 9-anthryldiazomethane (ADAM) [13], 4-bromomethyl-7-methoxycoumarin (Br-Mmc) [14] 1-bromoacetylpyrene (BAP) [15], and 1-pyrenyldiazomethane (PDAM) [16], bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone (BrDMEQ) [17].

DSP toxins are produced by several species of dinoflagellates, particularly *Dinophysis* and *Prorocentrum* spp. In addition to OA and DTXs, pectenotoxins (Fig. 1) have also been

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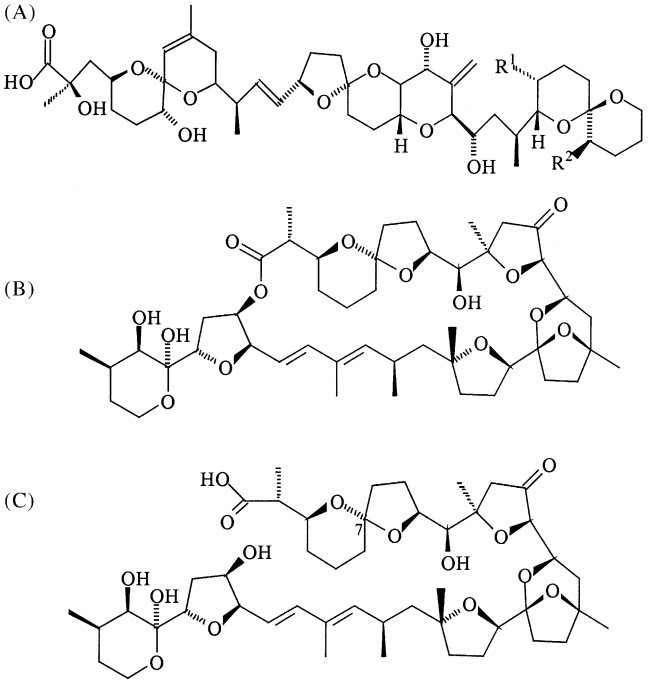

Fig. 1. (A) Okadaic acid (OA); R_1 = Me, R_2 = H; dinophysistoxin-1 (DTX1); R_1 = R_2 = Me, dinophysistoxin-2 (DTX2); R_1 = H, R_2 = Me; (B) pectenotoxin-2; (C) pectenotoxin-2 seco acids; PTX2SA, 7-*epi*-PTX2SA.

identified in *Dinophysis* spp. [18]. The large lactone ring of PTX2 is readily opened in mussel tissues to produce PTX2 seco acids [19]. Since PTX2 is not detectable using LC-FLD, due to the lack of a carboxylic acid functionality, it has been less frequently reported compared with the acidic DSP toxins. Toxin determination, using multiple tandem MS, is a very selective method which is not limited to the presence of characteristic functional moieties in the target analytes.

The development of liquid chromatography–mass spectrometry (LC–MS) has revolutionised the analysis of marine toxins in phytoplankton and shellfish samples [20–24]. Most previous MS studies have used selected ion monitoring (SIM) but this methodology has disadvantages, especially the observation of false positive signals [25] and ion suppression effects that are possible when analysing biological matrices [26]. Liquid chromatography–multiple tandem mass spectrometry (LC–MS/MS) is a remarkably sensitive method for the determination of toxins in very small samples containing low analyte concentrations [27,28]. This method has been applied to unambiguously identify and quantify DSP toxins in shellfish and phytoplankton and to confirm that PTX2 is produced by *D. fortii* [18] and that DTX2 is produced by *D. acuta* [29]. Many of the previous LC–MS methods for DSP toxins have been developed using positive ion mode. One disadvantage of this is that multiple adduct ions are often observed in the positive full scan MS and the occurrence of multiple ions can have serious implications for quantitation and reproducibility.

The co-occurrence of toxins from different classes in the same sample, and the poor availability of reference standard toxins, present difficulties to the analyst in the development of appropriate MS methods. The primary aim of this study was to develop a rapid, robust and sensitive method for the determination of OA, DTXs and PTXs in both shellfish and phytoplankton using LC–MS/MS with minimal sample preparation.

2. Experimental

2.1. Reagents and standard toxins

For sample extraction, general purpose grade solvents (methanol, chloroform, hexane) were used, while HPLC grade solvents (methanol, water, acetonitrile) were used during LC–MS/MS analysis; all solvents were purchased from Labscan (Dublin, Ireland). Spectroscopic grade trifluoroacetic acid (TFA) and analytical reagent grade ammonium acetate were purchased from Sigma–Aldridge (Dublin, Ireland). Purified DTX2 standard was prepared by isolation from bulk algae containing *D. acuta*, essentially as described previously [30]. Okadaic acid (98%), was purchased (Alexis, Birmingham, UK) and stored in methanol at −20 °C. The concentrations of OA and DTX2 solutions in methanol were verified using a certified standard solution of OA (25.3 μg/ml) obtained from the National Research Council (Halifax, Canada). PTX2, PTX2SA and 7-*epi*-PTX2SA standards were prepared by isolation from algae [31]. The isomer, PTX2SAi, cannot be purified to homogeneity as it is unstable and readily transforms to 7-*epi*-PTX2SA [32].

2.2. Mussel collection and preparation

Mussels (*Mytilus edulis*), containing ca. 40–50 g tissues, were collected from two locations, Bantry Bay, southwest Ireland and Sognefjord, southwest Norway. A portion (ca. 6 g) of homogenised shellfish hepatopancreas was extracted with methanol–water (4:1, v/v) (12 ml) and, after centrifugation, an aliquot (2.5 ml) of the supernatant was washed with hexane (2 ml × 2.5 ml). The upper layer was removed each time and water (1 ml) was added to the residual solution, which was extracted with chloroform (2 ml × 4 ml). This chloroform extract was evaporated to dryness under nitrogen and reconstituted in methanol (3 ml) and an aliquot (5 μl) was injected into the LC–MS.

2.3. Bulk phytoplankton collection and preparation

Phytoplankton samples were collected from the coastal areas of County Cork, Ireland. The plankton nets were 1.5 m in length, with mesh sizes of 27–100 μm. A portion of the collected algae (25–50 ml) was combined with an equivalent volume of methanol. To rupture cells, this mixture was alternatively sonicated and homogenised (IKA Ultra Turrax T25, Staufen, Germany) for 15 min and centrifuged. The supernatant was washed twice with equivalent volumes of

hexane. The aqueous methanol solution was extracted twice with equivalent volumes of dichloromethane and this extract was evaporated to dryness. The residue was reconstituted in methanol (10 ml) and an aliquot (5 μl) was analysed by LC–MS.

2.4. Picked cell sample preparation

Using an inverted microscope, individual *Dinophysis acuta* cells were picked and transferred to a vial. The samples, typically comprising 200–400 cells in 30% saline solution (1 ml), were mixed with methanol (1 ml), sonicated and filtered (0.45 μm). The aqueous methanol solution was washed with hexane (2 ml × 2 ml) and toxins were extracted with dichloromethane (3 ml × 2 ml). This extract was evaporated to dryness under nitrogen and reconstituted in methanol (50 μl). An aliquot (5 μl) was analysed by LC–MS.

2.5. Liquid chromatography–multiple tandem mass spectrometry (LC–MS/MS)

Toxin analysis was carried out using an API 3000 triple quadrupole mass spectrometer (Applied Biosystems) with a turbo assisted ionspray source. This was interfaced with an HP 1100 series liquid chromatograph (Agilent, Palo Alto, CA, USA). The instrumentation was controlled using Analyst v.1.2 software. The mobile phases used were: (a) water with 1.0 mM ammonium acetate; (b) acetonitrile with 1.0 mM ammonium acetate. Chromatographic separation of OA, DTXs, PTX2 and PTX2SAs was achieved using gradient elution, as described in the results and discussion section and in Table 1, on a reversed phase column (Luna C-18(2), 150 mm × 2.1 mm, 5 μm, Phenomenex, UK), at 35 °C using a flow rate of 0.2 ml/min. The autosampler temperature was 4 °C. The eluent flow was diverted to waste for 1.5 min after sample injection and MS detection was carried out in the 2–12 min period of the chromatography, followed by a second divert to waste prior to the next chromatographic sequence.

The mass spectrometer was operated in negative mode for the detection of OA, DTXs and PTXs using a TurboIonspray source set to 450 °C. The MS was tuned using an OA standard (1 μg/ml) and a phytoplankton sample containing OA, DTX2, PTX2 and PTX2SAs. The monitored ions were the $[M - H]^-$ precursor ions of OA, DTX2, PTX2, PTX2SAs and DTX1 at *m/z* 803, 803, 875, 857, 875 and 817, respectively and the most abundant product ion observed for each toxin. An optimised multiple reaction monitoring (MRM) experiment was established for the concurrent determination of the aforementioned toxins using the following conditions: ionspray voltage (IS) −4000 V, nebuliser gas (NEB) 10, curtain gas (CUR) 12, collision gas (CAD) 5, declustering potential (DP) −90 V, focusing potential (FP) −400 V, entrance potential (EP) −15 V, and cell exit potential (CEP) −13 V. The optimised collision energy (CE) was set to −70 V for the Q1/Q3 pairs, 803/255 (OA and DTXs), 817/255 (DTX1) and 857/137 (PTX2) and −65 V for the Q1/Q3 pairs, 876/137 (PTX2SAs). The MRM was performed with low resolution to achieve highest sensitivity and all Q1/Q3 pairs had a dwell time of 100 ms. Pectenotoxin standards, PTX2 and 7-*epi*-PTX2SA, were not available in sufficient amounts to obtain full calibration data using spiking experiments but were used in spectral studies and to confirm toxin identity in shellfish and phytoplankton samples.

Table 1
LC gradient programme

Time	A solvent	B solvent
0.0	60	40
0.5	55	45
0.51	25	75
6.0	25	75
6.01	60	40
12.0	60	40

3. Results and discussion

3.1. Multiple reaction monitoring (MRM) mass spectrometry

An improved method for the simultaneous identification and quantitation of DSP toxins in shellfish and phytoplankton has been developed and validated. A quantitative LC–MS/MS assay for the analysis of biological samples generally consists of three stages, sample preparation, chromatographic separation and MS–MS detection [33]. A gradient liquid chromatography method was developed linked with multiple tandem mass spectrometry (LC–MS/MS). Ionisation in negative mode generated the $[M - H]^-$ ion for each toxin. To achieve optimum sensitivity and selectivity, multiple reaction monitoring (MRM) was implemented. The fragmentation of the target toxins was optimised to efficiently generate a predominant product ion from each precursor ion. For example, the selected precursor/product ion combinations (Q1/Q3 pairs) were 803/255 for OA and DTX2 and were 857/137 for PTX2. Four MRM scan events were implemented to simultaneously determine OA, DTX2, DTX1, PTX2 and PTX2SAs in extracts of marine phytoplankton and mussel samples, collected from the south-west coast of Ireland, and in mussel extracts from Norway.

One advantage of using negative ionisation is the generation of a single molecule-related ion, $[M - H]^-$. Although positive mode can also be used, several molecule-related ions can be produced. In addition to the $[M + H]^+$ ion, sodium and ammonium adducts, $[M + Na]^+$ and $[M + NH_4]^+$, are usually formed which results in a loss of sensitivity and poor reproducibility. It was found that the detection sensitivity for all of the toxins studied was better in negative rather than in positive mode and this is partly

due to the greater difficulty in fragmentation of sodiated adducts.

3.2. Chromatographic separation of polyether DSP toxins

An LC-gradient was required for the separation of DSP toxins due to the large differences in polarity between the toxins. OA, DTXs and PTX2SAs are acids but PTX2 is a lactone and therefore significantly less polar, eluting much later in isocratic LC. The optimised elution conditions are shown in Table 1. Owing to the high selectivity of MRM, chromatographic resolution is not essential for toxins with different Q1/Q3 ion pairs. However, MRM cannot distinguish between isomers that have identical product ion spectra and, therefore in the method described here, chromatographic resolution was essential. A chromatographic run of 12 min allowed sufficient resolution of all toxins, including the isomers, OA/DTX2 and PTX2SAi and 7-*epi*-PTX2SA (Fig. 1), and the method was found to be reproducible with respect to the different matrices.

Analysis of trace analytes in biological matrices can be susceptible to interferences arising from ion suppression and this is particularly problematic with single stage MS. A recent LC–MS study using single ion monitoring (SIM) demonstrated interferences in the analysis of DSP toxins, and standard addition was necessary to compensate for this problem [26]. Therefore, the possibility of matrix interferences contributing to ion suppression in the LC–MS/MS method described here was investigated. Experiments were designed to determine if there were segments in the chromatography where matrix interferences could result in significant ion suppression. This involved chromatography of toxin-free mussel extracts whilst continuously infusing an OA standard solution (1 μg/ml), at a flow rate of 50 μl/min. The shaded regions in the chromatogram (Fig. 2) are where DSP toxins typically elute. Since no significant variation in the OA signal was observed, it can be concluded that matrix components from mussel and phytoplankton extracts are not important.

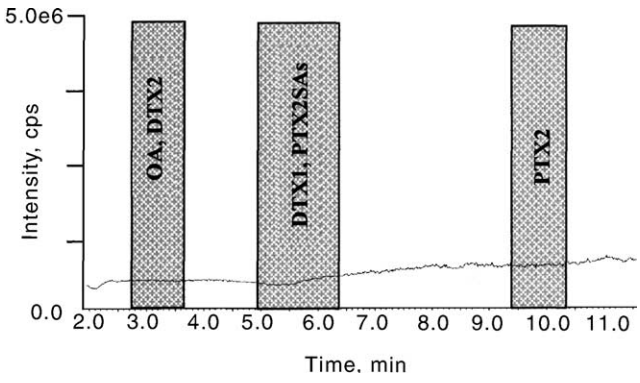

Fig. 2. Signal output from the chromatography of a blank mussel (*M. edulis*) extract whilst infusing OA solution. The regions where toxins typically elute are shaded.

3.3. LC–MS/MS calibration data for DSP toxins in shellfish

Optimisation of the parameters in the triple-stage quadrupole MS detector and subsequent calibration studies were carried out using a certified OA standard. For shellfish studies, a certified reference mussel material containing OA was used for method development. Calibration studies

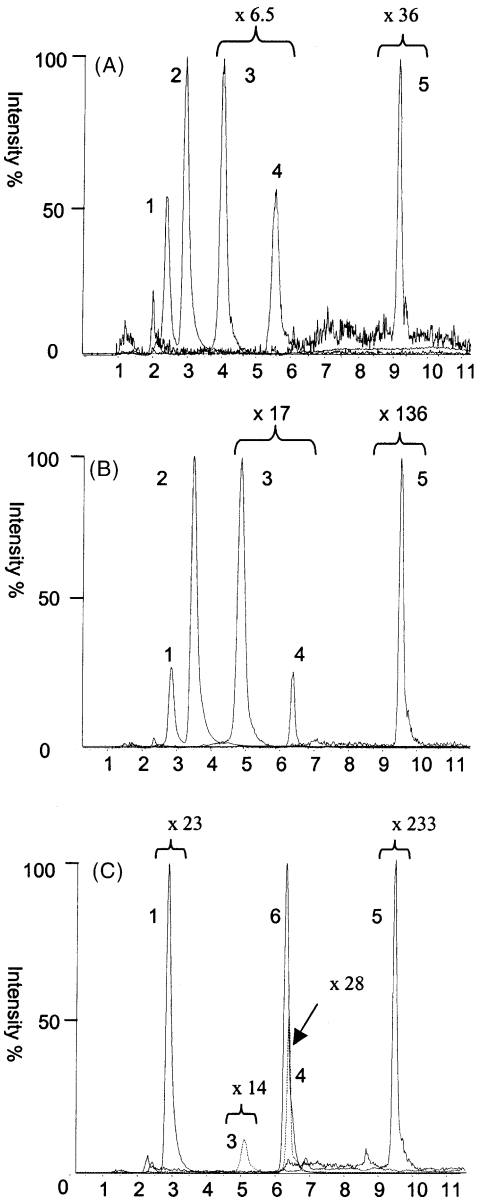

Fig. 3. MRM chromatograms: (A) bulk algae sample; (B) Irish mussels (*M. edulis*); (C) Norwegian mussels (*M. edulis*): (1) OA (2.8 min); (2) DTX2 (3.5 min); (3) PTX2SAi (4.9 min); (4) 7-*epi*-PTX2SA (6.5 min); (5) PTX2 (9.8 min); (6) DTX1 (6.65 min) (signal amplification, if any, is shown above each peak). LC conditions: a gradient of acetonitrile–water containing 1 mM ammonium acetate was used (see Table 1), at 35 °C, with a flow rate of 0.2 ml/min; the column was a Luna C-18(2) (150 mm × 2.1 mm, 5 μm, Phenomenex). The selected MRM (Q1/Q3 pairs) were: 803/255 (OA and DTX2), 876/137 (PTX2SAs), 817/255 (DTX1), 857/137 and (PTX2).

were carried out using certified OA standard dissolved in methanol. The LC–MS/MS calibration was linear in the range 0.002–1.7 μg/ml with a good correlation coefficient (r^2 = 0.9964, N = 3) over three days. The detection limit (signal/noise = 3) was <1 pg OA on-column. The LC–MS/MS calibration, using spiked OA in mussel hepatopancreas tissues, was linear in the range 0.007–1.00 μg/ml with a good correlation coefficient (r^2 = 0.9993, N = 3). Good reproducibility data were also achieved with %RSD values (N = 3) ranging from 3.0% (0.65 μg OA/ml) to 5.8% (0.065 μg OA/ml), for shellfish extracts, which is equivalent to 1.5 and 0.15 μg/g shellfish tissue, respectively. Using the protocol presented here, the detection limit was equivalent to 0.48 ng/g shellfish tissue. The LC–MS/MS calibration, using spiked DTX2 in shellfish tissue, was linear in the range 0.054–8.5 μg/ml, with a good correlation coefficient (r^2 = 0.9992, N = 3). Good reproducibility data were also achieved with %RSD values (N = 3) ranging from 3.2% (0.56 μg DTX2/ml) to 5.7% (0.14 μg DTX2/ml), for shellfish extracts. These values are equivalent to 1.2 and 0.32 μg/g shellfish tissue, respectively.

3.4. DSP toxin profiles in phytoplankton and mussels

Fig. 3 shows MRM chromatograms from three extracts: (A) bulk phytoplankton; (B) mussels (*M. edulis*) from Ireland; and (C) mussels (*M. edulis*) from Norway. Five DSP toxins were identified in phytoplankton and mussels from Ireland, OA, DTX2, PTX2SAi, 7-*epi*-PTX2SA and PTX2. The predominant toxin in all Irish samples was DTX2. Although PTX2SA is the predominant pectenotoxin in New Zealand shellfish [31], it was not detected in any of the samples in this study. Significant levels of OA were also found but high levels of PTX2 (up to 0.57 μg/ml) were only detected in phytoplankton samples. Table 2 shows the toxin profiles for shellfish and phytoplankton collected from southwest Ireland (2001) and Norway (1998). These toxin profiles differ significantly to those reported in New Zealand phytoplankton containing *D. acuta* in which DTX2 was never found [34].

The most striking differences between the profiles of mussels from Ireland and Norway were the absence of DTX2 from Norwegian mussels and the high levels of DTX1 in the latter.

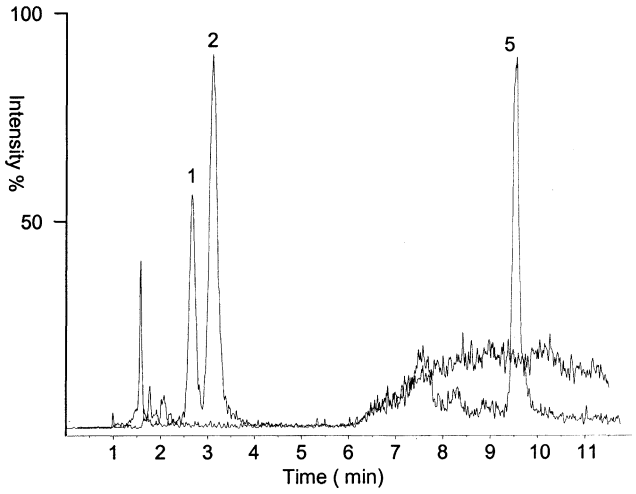

Fig. 4. MRM chromatogram from an extract of 20 picked cells of *Dinophysis acuta* (chromatographic conditions as in Fig. 3).

DTX1 was not detected in any Irish samples. Generally, the levels of PTX2SAs in mussels were much higher than PTX2 and this is expected since it has previously been demonstrated that rapid bioconversion of PTX2 to the corresponding seco acids occurs in mussels [19].

3.5. Analysis of monocultures of D. acuta cells

The LC–MS/MS method was also applied to the analysis of *D. acuta* cells that were hand picked from a phytoplankton sample. This sample contained ca. 60–70% *D. acuta* and the remaining phytoplankton cells were comprised mainly of non-toxic *Ceratium* spp. as well as small amounts of *Protoperidinium* spp. A consequence of the very high sensitivity of this MRM method is that only a small number of cells (n = 20–40) were necessary to obtain toxin profiles. The average toxin content was in the range 25–189 pg total DSP toxins/cell. Fig. 4 shows an MRM chromatogram that was obtained using an extract of 12 *D. acuta* cells; OA (7.0 pg/cell), DTX2 (11 pg/cell) and PTX2 (7.2 pg/cell). PTX2SAs were not detected in this sample and this indicates that these toxins are probably not present in intact *D. acuta* cells.

Table 2
DSP toxin profiles in mussels and phytoplankton

Samples	OA	DTX2	PTX2SAi	7-*epi*-PTX2SA	PTX2	DTX1
Ireland (mean)	1.5	3.7	0.29	0.064	0.068	ND
M. edulis (range; N = 7)	0.07–8.2	0.3–15	0–0.55	0.045–0.12	0–0.1	
Ireland (mean)	0.23	0.40	0.01	0.009	0.20	ND
D. acuta (range; N = 7)	0.01–0.56	0.03–0.57	0–0.026	0–0.018	0.003–0.57	
Norway (mean)	1.0	ND	0.39	1.5	0.14	18
M. edulis (range; N = 4)	0.59–1.7		0.12–0.74	0.31–3.1	0.08–0.29	12–26

Concentration values; *M. edulis* (μg/g). *D. acuta* in seawater (μg/ml); not detected (ND).

4. Conclusion

An LC–MS/MS method has been developed which permits the rapid, unambiguous identification and quantitation of OA, DTX1, DTX2, PTX2 and PTX2SAs, in shellfish and phytoplankton samples. The method requires minimal sample clean-up, no pre-concentration is necessary and is readily automated. This highly sensitive method allows the determination of DSP toxin profiles in 12–20 phytoplankton cells. The lack of matrix interferences using this LC–MS/MS method for DSP toxin analysis in shellfish should make it amenable to the determination of these toxins in a range of biological samples.

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