

# Amnesic shellfish poisoning toxins in bivalve molluscs in Ireland

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## Abstract

In December 1999, domoic acid (DA) a potent neurotoxin, responsible for the syndrome Amnesic Shellfish Poisoning (ASP) was detected for the first time in shellfish harvested in Ireland. Two liquid chromatography (LC) methods were applied to quantify DA in shellfish after sample clean-up using solid-phase extraction (SPE) with strong anion exchange (SAX) cartridges. Toxin detection was achieved using photodiode array ultraviolet (LC-UV) and multiple tandem mass spectrometry (LC-MS<sup>n</sup>). DA was identified in four species of bivalve shellfish collected along the west and south coastal regions of the Republic of Ireland.

The amount of DA that was present in three species was within EU guideline limits for sale of shellfish (20 µg DA/g); mussels (*Mytilus edulis*), <1.0 µg DA/g; oysters (*Crassostrea edulis*), <5.0 µg DA/g and razor clams (*Ensis siliqua*), <0.3 µg DA/g. However, king scallops (*Pecten maximus*) posed a significant human health hazard with levels up to 240 µg DA/g total tissues. Most scallop samples (55%) contained DA at levels greater than the regulatory limit. The DA levels in the digestive glands of some samples of scallops were among the highest that have ever been recorded (2820 µg DA/g).

**Keywords:** ASP; Domoic acid; Shellfish toxin; Food safety; LC-MS

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## 1. Introduction

Domoic acid (DA) was first identified in the seaweed, *Chondria armata*, in Japan (Takemoto and Daigo, 1960) but achieved notoriety as the toxin responsible for a serious seafood poisoning incident in Canada in 1987. The illness occurred following the consumption of mussels (*Mytilus edulis*) when more than 100 people were hospitalised with at least four fatalities (Bird and Wright, 1989). The human symptoms included persistent short-term memory impairment in some patients which prompted the naming of this

toxic syndrome as Amnesic Shellfish Poisoning (ASP) (Bird and Wright, 1989; Perl et al., 1990; Todd, 1993). DA levels in mussels were up to 900 µg DA/g (Todd, 1993). The primary sources of DA in bivalve shellfish are the pinnate diatoms, *Pseudonitzschia* spp., and toxin accumulates in filter-feeding shellfish that graze on these phytoplankton (Bates et al., 1989; Martin et al., 1990; Fritz et al., 1992; Dortch et al., 1997; Amzil et al., 2001). DA has been identified in cultures of *Pseudonitzschia australis* that were obtained from southwest Ireland (Cusack et al., 2002). Although DA (Fig. 1) was identified as the primary toxin responsible for ASP, other DA isomers have since been found in shellfish (Wright et al., 1990).

Following the Canadian toxic outbreak, DA has been detected in a variety of shellfish throughout the world. Reports of DA in shellfish have included USA (Horner et al.,

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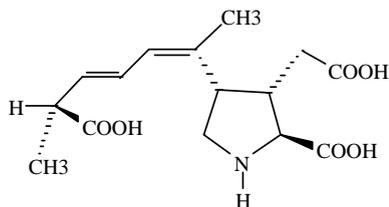


Fig. 1. Structure of domoic acid (DA).

1993), New Zealand (Rhodes et al., 1995), Mexico (Beltran et al., 1997) and several European countries (Miguez et al., 1996; Vale and Sampayo, 2001; Amzil et al., 2001; Gallacher et al., 2001). The environmental impacts of this toxin has also included large-scale animal mortalities involving birds (Fritz et al., 1992; Work et al., 1993; Beltran et al., 1997), sea-lions (Sholin et al., 2000; Gulland et al., 2002) and whales (Lefebvre et al., 2002).

A regulatory level of 20  $\mu\text{g DA/g}$  in total tissues has been widely adopted as a limit for sale of shellfish. Several chromatographic methods have been developed for the quantitative determination of DA in shellfish and marine phytoplankton. Liquid chromatography with photodiode-array ultraviolet detection (LC-UV) can be used following strong anion exchange solid phase extraction (SPE) clean-up of shellfish extracts (Quilliam et al., 1989a, 1995; Lawrence et al., 1994). Three fluorescent reagents, 9-fluorenylmethylchloroformate (FMOC), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) and 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), have been used to derivatise DA in phytoplankton and seawater (Pocklington et al., 1990; Sun and Wong, 1999; James et al., 2000), prior to chromatography and fluorimetric detection (LC-FLD).

Several mass spectrometric (MS) techniques have also been employed to determine DA in shellfish, including LC-MS (Quilliam et al., 1989b; Thibault et al., 1989; Lawrence et al., 1994; Hess et al., 2001) and capillary electrophoresis-MS (Gago-Martinez et al., 2003). It has been demonstrated that DA can be determined with high sensitivity and selectivity using LC-MS<sup>3</sup> with an ion-trap instrument (Furey et al., 2001) and LC-MS/MS using a triple quadrupole instrument (Holland et al., 2003). We now report studies to determine the extent of DA contamination of four species of bivalve shellfish in Ireland and the implications for food safety.

## 2. Materials and method

### 2.1. Materials

Purchased chemicals included trifluoroacetic acid (Aldrich, Dorset, UK) and formic acid (Merck, Darmstadt, Germany). All solvents were HPLC grade and were purchased from Labscan (Dublin, Ireland). MUS-1B, certified reference material ( $38.3 \pm 0.8 \mu\text{g/g}$ ; DA in mussel

tissues) and DACS-1C, certified calibration solution (100  $\mu\text{g DA/ml}$ ), were purchased from the National Research Council (NRC), Halifax, Canada. Cultivated shellfish, mussels (*Mytilus edulis*), oysters (*Crassostrea gigas*), king scallops (*Pecten maximus*) and razor clams (*Ensis siliqua*), were collected from the coastal regions of Ireland.

### 2.2. Sample preparation

Shellfish preparation was performed using a procedure similar to that previously described (Quilliam et al., 1995). Shellfish tissues (30–50 g) were homogenised and approximately 4 g of this homogenate was accurately weighed. Methanol–water (1:1, 16 ml) were added and homogenised (3 min), the resulting slurry was then centrifuged at 3000g for 10 min and the supernatant was filtered (0.45  $\mu\text{m}$ , Phenomenex, Macclesfield, UK). For sample clean-up, solid phase extraction (SPE) was performed using a strong anion exchange (SAX) cartridge (3 ml, J.T. Baker, Deventer, Holland), conditioned with methanol (6 ml), water (3 ml) and methanol–water (1:1, 3 ml). The filtered supernatant (5 ml) was loaded onto the cartridge which was washed with acetonitrile–water (1:9, 5 ml). The cartridge was eluted with formic acid solution (0.1 M). The dead volume (0.5 ml) was discarded and the eluent (3 ml) was collected for analysis by LC-UV and LC-MS<sup>3</sup>. For direct analysis using LC-MS<sup>3</sup>, shellfish extracts (1 ml) were diluted to 10 ml with water and a 5  $\mu\text{l}$  aliquot was injected.

### 2.3. Liquid chromatography-ultra violet (LC-UV)

LC-UV analysis was carried out using an aliquot (10  $\mu\text{l}$ ) from the SPE stage 2.2. The LC system was an HP 1100 (Agilent, Cheshire, UK) which consisted of a binary pump, a thermostatically controlled autosampler (4 °C) and a UV photodiode array detector. Gradient chromatography was performed using acetonitrile–water (5–40% acetonitrile in 25 min) containing 0.05% trifluoroacetic acid (TFA), at a flow rate of 0.2 ml/min, with a reversed phase column, C<sub>18</sub> Luna (2), 5  $\mu\text{m}$ , 150  $\times$  2.0 mm, Phenomenex) at 40 °C.

### 2.4. Liquid chromatography-multiple tandem mass spectrometry (LC-MS<sup>n</sup>)

LC-MS<sup>n</sup> analysis was carried out using a quadrupole ion-trap mass spectrometer (LCQ, ThermoFinnigan, San Jose, CA, USA). The LC method was similar to that used in Section 2.3 except that isocratic elution was used, acetonitrile–water (60–40). The MS parameters were essentially as described previously (Furey et al., 2000). The MS<sup>3</sup> target ion sequence was  $m/z$  312  $\rightarrow$  266  $\rightarrow$  248.

### 3. Results and discussion

Domoic acid (DA) was identified for the first time in Irish shellfish during the winter of 1999, and a study was undertaken to examine the potential impact of this toxin on food safety. The four most commercially important bivalve shellfish species in Ireland, mussels (*M. edulis*), oysters (*C. gigas*), king scallops (*P. maximus*) and razor clams (*E. siliqua*), were studied throughout the entire coastal region of Ireland. Two chromatographic methods were employed for the quantitation of DA in shellfish. These methods utilised liquid chromatography with photodiode array ultraviolet detection (LC-UV) and electrospray ion-trap multiple tandem mass spectrometry (LC-MS<sup>n</sup>).

#### 3.1. Determination of DA in shellfish using LC-UV

Sample preparation and analysis of DA in shellfish was performed using a procedure similar to that previously described (Quilliam et al., 1995). Exclusive reliance on LC-UV for the determination of DA in crude shellfish extracts is inadvisable as false-positive results may be produced (Hess et al., 2001) and tryptophan interference is also common (Lawrence et al., 1994). A strong anion exchange solid phase extraction (SPE) has been recommended for sample clean-up of extracts from mussels (Quilliam et al., 1995) and scallops (Gallacher et al., 2001). The average recovery of DA from the reference standard mussel material, MUS-1B, using SPE, was 92% ( $n=5$ ). However, it was observed that the recovery of DA from scallop tissues was inconsistent and studies revealed that the recovery was dependent on the concentration of DA. The data from this SPE study are summarised in Fig. 2 and it was shown that there was reduced extraction efficiency at higher levels of DA. The average DA recovery ( $n=5$ ) from scallop hepatopancreas was 97%, at a concentration of 20  $\mu\text{g DA/g}$ , but diminished to 79% at 800  $\mu\text{g DA/g}$ . It is necessary therefore to retest sample extracts that had higher levels of DA using LC-MS without SPE to obtain reliable quantitative data. A representative chromatogram (LC-UV) from an

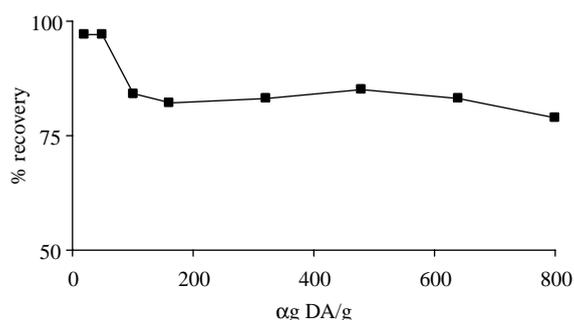


Fig. 2. Recovery of domoic acid (DA) from extracts of scallop (*P. maximus*) digestive glands using anion exchange solid phase extraction (SPE).

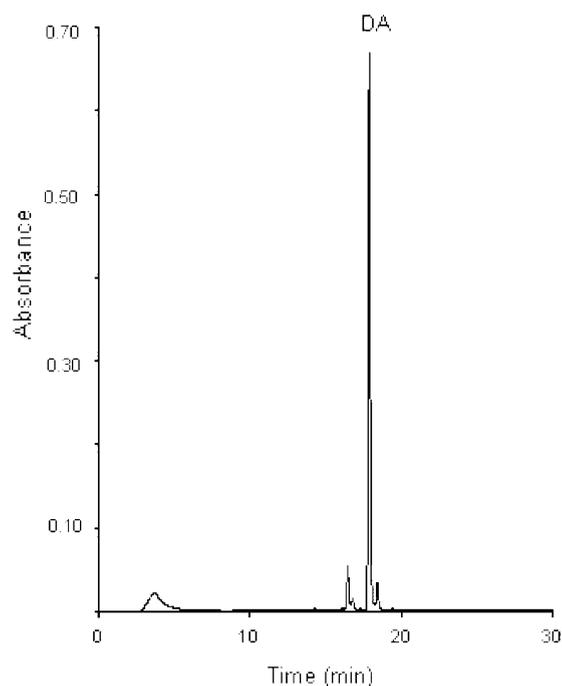


Fig. 3. Chromatogram from the LC-UV/DAD analysis of DA in scallop (*P. maximus*) total tissue. The concentration of domoic acid in the scallop total tissue was 25  $\mu\text{g/g}$  (see experimental section for chromatographic conditions).

extract of scallop (total tissues) shows DA as the main component with several minor isomers, isodomoic acid and *epi*-domoic acid (Fig. 3).

#### 3.2. Determination of DA in shellfish using LC-MS<sup>n</sup>

A quadrupole ion-trap mass spectrometer (MS) was used to confirm DA in shellfish tissues (Furey et al., 2001). The MS was equipped with an electrospray ion-spray (ESI) interface and operated in positive ion mode. The molecule-related ion,  $[\text{M}+\text{H}]^+$ , for DA at  $m/z$  312, was trapped and fragmented; two prominent ions were observed in the MS<sup>2</sup> spectrum (Fig. 4a), at  $m/z$  266 and  $m/z$  294. The ion at  $m/z$  266 ion was formed by the loss of a formic acid molecule  $[\text{M}+\text{H}-\text{HCOOH}]^+$  or by the loss of water and carbon monoxide  $[\text{M}+\text{H}-\text{H}_2\text{O}-\text{CO}]^+$ . This ion was also trapped and fragmented to produce the MS<sup>3</sup> spectrum (Fig. 4b) in which the prominent ions were at  $m/z$  248,  $m/z$  220 and  $m/z$  193. The latter ion is characteristic of DA and represents the disruption of the proline ring (Furey et al., 2001). A feature of quadrupole ion-trap instruments is the improvement in detection sensitivity in multiple MS modes, which is attributed to the reduction in background noise in MS<sup>2</sup> and MS<sup>3</sup> stages (James et al., 2003). A certified reference mussel material containing DA (MUS-1B) was used for method development. Linear calibrations were obtained using shellfish extracts with 0.025–10  $\mu\text{g DA/ml}$

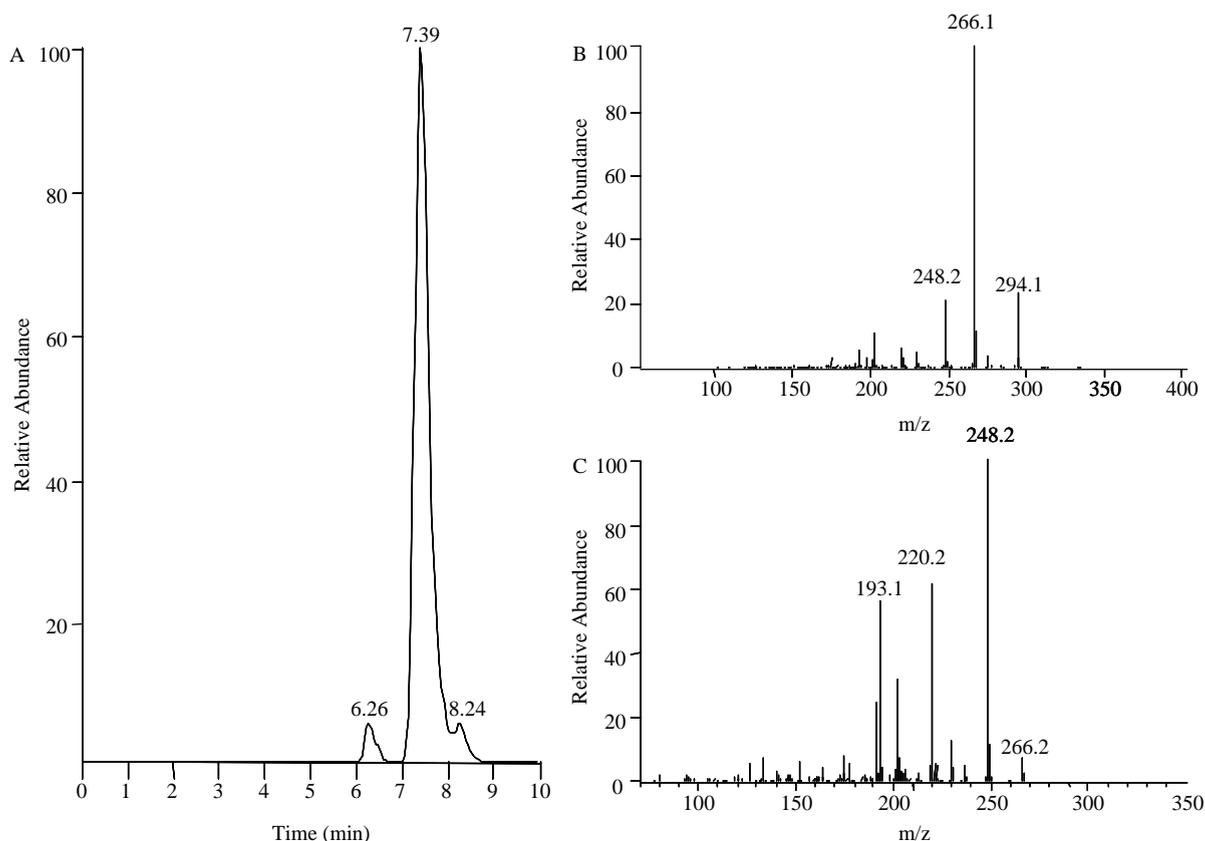


Fig. 4. (A) Chromatogram of an extract from scallop (*P. maximus*) digestive glands with detection using quadrupole ion-trap mass spectrometry (MS). The retention time of DA was 7.39 min and the other peaks are DA isomers (see experimental section for chromatographic conditions). (B) The MS<sup>2</sup> spectrum generated by targeting the *m/z* 312 ion of DA. (C) The MS<sup>3</sup> spectrum generated by targeting the *m/z* 312 and *m/z* 266 ions of DA.

( $r^2 = 0.9994$ ). Using the LC-MS<sup>3</sup> method, it was possible to analyse crude shellfish extracts containing a wide range of DA concentration levels, without sample pre-treatment. The sample spectra (Fig. 4) were obtained from an extract of the digestive glands of scallops harvested from Co. Galway (Fig. 5, no. 3).

### 3.3. Domic acid in multiple shellfish species

In December 1999, this research group first detected DA in scallops (*P. maximus*) and the toxin levels found were up to 160 µg DA/g total tissues (eight times the regulatory limit). As a result of this toxic event, a programme was established to monitor DA in shellfish for a period of 6 months. The main cultivated bivalve molluscs that were studied included, mussels (*M. edulis*), oysters (*C. gigas*), razor clams (*E. siliqua*) and scallops (*P. maximus*). During this period of monitoring, DA was detected at trace levels in razor clams, mussels and oysters. However, the species most affected were scallops, with 55% of samples exceeding

the regulatory limit of 20 µg DA/g. Table 1 summarises the data from this monitoring programme of DA in Irish shellfish. A previous publication reported that the razor clam, *Siliqua patula*, could accumulate DA at significant levels. A high proportion (42%) of samples from the Pacific coastline of the USA in 1991–1993 were found to contain DA in excess of 20 µg/g (Altwein et al., 1996) and DA contamination in razor clams continued to be of concern in this region (Wekell et al., 2002). Therefore, locally produced razor clams (*E. siliqua*) were initially examined for DA but toxin levels were consistently low (0.09–1.3 µg/g total tissue).

Mussels (*M. edulis*) were responsible for the initial fatal outbreak of ASP in Canada and very high levels of DA (up to 350 µg/g) were found in samples from Prince Edward Island (Gilgan et al., 1990). The situation with Irish mussels (*M. edulis*) was somewhat different with only 2% of samples testing positive for DA and as the toxin levels were less than 0.90 µg DA/g in mussels, this did not constitute a threat to human health. In addition, DA toxicity in oysters (*C. gigas*)



Fig. 5. Map of Ireland showing areas where DA was detected in shellfish. Site no. 1: Bantry Bay; no. 2: Sneem; no. 3: Bertraghbouy Bay.

also proved to be insignificant (Table 1). However, DA was detected in 89% of scallops (*P. maximus*) tested throughout this survey, with levels in the range of 0.05–240 µg DA/g total tissue. Fig. 5 shows the main locations along the Irish coast where shellfish tested positive for DA. Although these results revealed a wide geographical distribution of DA in shellfish in Ireland, only scallops were seriously affected, with 55% of samples containing toxin in excess of the regulatory limit.

Table 1  
Domoic acid (DA) in shellfish cultivated in Ireland (Dec. 1999–May 2000)

Species	Samples analysed	Positive <sup>a</sup> (%)	DA µg/g total tissues
Razor clams ( <i>E. siliqua</i> )	14	6 (43%)	0.09–0.66
Mussels ( <i>M. edulis</i> )	97	2 (2%)	0.09
Oysters ( <i>C. gigas</i> )	60	2 (3%)	0.27–0.90
Scallops ( <i>P. maximus</i> )	175	156 (89%)	0.05–240 <sup>b</sup>

<sup>a</sup> >0.01 µg DA/g.

<sup>b</sup> 55% of scallop (*P. maximus*) samples exceeded the regulatory limit of 20 µg DA/g total tissues.

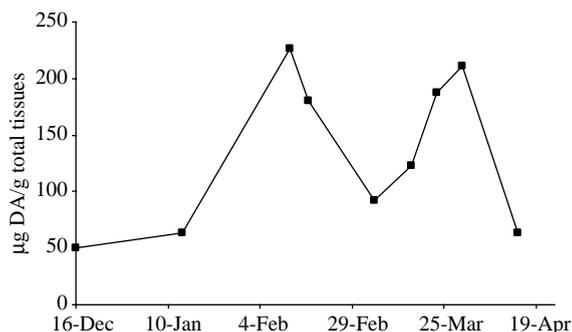


Fig. 6. Variation in the levels of DA in the digestive glands (hepatopancreas) of scallops from Sneem, Ireland (Fig. 5, no. 2). The toxin concentration range was 50–230 µg DA/g over a period of 6 months (Dec. 1999–May 2000).

#### 3.4. Temporal variation of domoic acid in scallops (*P. maximus*)

The temporal variation of DA in scallop tissues was surveyed for the period December–May 2000 (Fig. 6). The DA concentrations ranged from 50 to 230 µg/g and large fluctuations in toxin levels were observed during winter months. It has previously been demonstrated that DA in shellfish originates from *Pseudonitzschia* spp. (Bates et al., 1989; Martin et al., 1990), but only low levels of these diatoms were observed during this study. It was also observed that the intoxication levels in mussels and scallops were different in the same cultivation area. Thus, mussels, cultivated in Bantry Bay (Fig. 5, no. 1) using ropes suspended in deep waters, consistently did not contain detectable levels of DA, whilst scallops, cultivated along the shore line, had very high levels of toxin (up to 2270 µg DA/g hepatopancreas). An examination of the sediments from scallop cultivation areas did not reveal significant levels of DA. It is difficult to explain the large variations in the levels of DA in scallops during periods when populations of *Pseudonitzschia* spp. were consistently low.

## 4. Conclusion

This study, conducted following the first discovery of DA in Ireland, revealed large geographical, temporal and species variations of DA in bivalve molluscs. The cultivation areas studied were mainly along the west coast of Ireland. DA levels were at their highest during winter months despite the absence of significant levels of *Pseudonitzschia* spp. The bivalve mollusc species most affected by DA was the king scallop (*P. maximus*), where the toxin was found predominantly in the hepatopancreas. However, the adductor muscle of scallops did not contain dangerous levels of DA. Only trace levels of DA were present in mussels (*M. edulis*), oysters (*C. gigas*) and razor

clams (*E. siliqua*) and these species pose a lower food safety risk than scallops.

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