ELSEVIER

Journal of Chromatography A, 749 (1996) 33–40

JOURNAL OF CHROMATOGRAPHY A

# Isolation of dinophysistoxin-2 and the high-performance liquid chromatographic analysis of diarrhetic shellfish toxins using derivatisation with 1-bromoacetylpyrene[1]

Seán S. Kelly, Alan G. Bishop, Eoin P. Carmody, Kevin J. James*

*Ecotoxicology Research Unit, Chemistry Department, Cork RTC, Bishopstown Cork, Ireland*

Received 12 December 1995; revised 26 February 1996; accepted 25 April 1996

**Abstract**

The rare diarrhetic shellfish toxin, dinophysistoxin-2 (DTX-2), was isolated from the digestive glands of mussels (*Mytilus edulis*). This was achieved by chromatography on silica and Sephadex LH-20 followed by reversed-phase solid phase extraction and semi-preparative high-performance liquid chromatography (HPLC) with an Ultremex $C_{18}$ column. Using 1-bromoacetylpyrene (BAP), as a precolumn derivatisation reagent, the diarrhetic shellfish toxins, okadaic acid (OA), dinophysistoxin-1 (DTX-1) and DTX-2, were determined by HPLC with fluorimetric detection. Derivatisation using BAP was compared with 9-anthryldiazomethane (ADAM) and, although the latter exhibited a four-fold better sensitivity, the BAP method gave fewer artefact peaks from reagent decomposition. The limits of detection of OA and DTX-2 were 0.4 ng on-column using BAP, which permits this method to be used for the regulatory control of these toxins in shellfish.

*Keywords:* Dinophysistoxin-2; Toxins; 1-Bromoacetylpyrene; Diarrhetic shellfish toxins

## 1. Introduction

Severe gastrointestinal disturbance can result from the ingestion of shellfish that are contaminated with toxins that originate from marine microalgae. Bivalve molluscs are particularly susceptible when feeding on toxigenic dinoflagellates such as *Dinophysis* and *Prorocentrum* spp. [1]. The syndrome is called diarrhetic shellfish poisoning (DSP) and it has occurred with increasing frequency in recent years. The polyether carboxylic acids, okadaic acid (OA) and dinophysistoxin-1 (DTX-1) (Fig. 1) have been identified as the toxins responsible for most outbreaks of DSP and this topic has been the subject of a recent review [2]. These compounds exert their bioactivity by the potent inhibition of protein phosphatases, PP1 and PP2A [3]. It is this activity that has led to the use of OA as a valuable reagent for

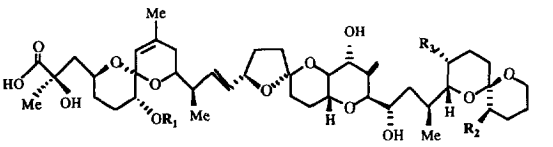


Fig. 1. Structures of diarrhetic shellfish toxins. Okadaic acid (OA), $R_1$=H, $R_2$=H, $R_3$=$CH_3$; dinophysistoxin-1 (DTX-1), $R_1$=H, $R_2$=$CH_3$, $R_3$=$CH_3$; dinophysistoxin-2 (DTX-2), $R_1$=H, $R_2$=$CH_3$, $R_3$=H; dinophysistoxin-3 (DTX-3), $R_1$=acyl.

*Corresponding author.

[1]Presented at 19th International Symposium on Column Liquid Chromatography (HPLC'95), Innsbruck, Austria.

0021-9673/96/$15.00 
*PII* S0021-9673(96)00448-7

biochemical cellular studies and it is now commercially available. However, an isomer of OA, dinophysistoxin-2 (DTX-2), was recently identified [4] in mussels (*Mytulis edulis*), cultivated in Ireland, where it continues to be the predominant toxin [5]. Bioassays, with live rodents, are used by most regulatory authorities to screen for diarrhetic toxicity in shellfish but internationally agreed limits for individual toxins will depend on toxicity evaluations. The new toxin, DTX-2, was recently detected in dinoflagellates in Spain [6] and in shellfish from Portugal [7]. In this paper, we describe the isolation of this toxin from shellfish for use as a reference standard and for toxicity evaluations.

Fluorimetric HPLC analysis [8] of the 9-anthrylmethyl derivatives of the acidic diarrhetic toxins has become an established method and this protocol was recently subjected to critical examination [9]. The derivatising reagent, 9-anthrylmethyldiazomethane (ADAM, Fig. 2a), is somewhat unstable and this may give rise to interferences from artefact peaks in HPLC. A number of other derivatising reagents have been proposed for the determination of OA in shellfish, including N-(9-acridinyl)-bromoacetamide [10], 1-bromoacetylpyrene (BAP, Fig. 2b) [11], 1-pyrenyldiazomethane (PDAM) [12], 4-bromomethyl-7-methoxycoumarin (Br-Mmc) [13] and 2,3-(anthracenedicarboximido)ethyl trifluoromethanesulphonate (AE-OTf) [14]. Difficulties arise with each of these methods in determining trace analytes in the presence of large reagent peaks and artefacts. The application of column switching procedures with several of these reagents has recently been employed to aid automation [14,15]. In this paper, the application of BAP for the simultaneous determination of OA, DTX-1 and DTX-2 in shellfish is reported and compared with the ADAM method.

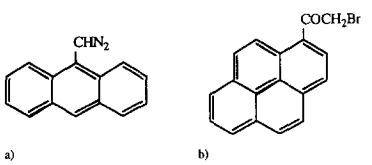


Fig. 2. (a) 9-anthryldiazomethane (ADAM) and (b) 1-bromoacetylpyrene (BAP).

## 2. Experimental

### 2.1. Instrumentation

The HPLC system consisted of a pump (LC-10AD), column oven (CTO-10A) and an RF-551 fluorescence detector (Shimadzu, Duisberg, Germany) with an autosampler (ISS-100, Perkin Elmer, Uberlingen, Germany). The analytical HPLC column used was an Ultremex $C_{18}$, 5$\mu$m, 250×3.2 mm (Phenomenex, Macclesfield, UK), with a precolumn (Ultremex $C_{18}$, 5$\mu$m, 30×3.2 mm) and an in-line filter (3 mm×0.5 $\mu$m, Rheodyne, Cotati, CA, USA). An Ultremex $C_{18}$, 5$\mu$m, 250×10 mm column with a precolumn, 60×10 mm (Phenomenex) were used for semi-preparative HPLC.

Solvent evaporation under nitrogen was carried out using a Turbo Vap LV evaporator (Zymark, Warrington, UK). Sample preparation required the following equipment: an homogeniser (Ultra-Turrax T25, Janke and Kunkel, Staufen, Germany), centrifuges (Beckman Model J2-21, High Wycombe, UK and Easyspin, Sorvall Instruments, Stevenage, UK), vortex-mixer (Maxi-Mix II, Thermolyne type 37600, Barnstead/Thermolyne, Dubuque, IA, USA) and a sonic bath (Sonicor SC-42, Sonicor Instrument, Copiague, NY, USA).

### 2.2. Software

Chromatographic data handling was performed using an Axxi-Chrom 717 chromatography data station (Axxiom Chromatography, Gloucester, UK). Data were transferred to Microsoft Excel for further graphical manipulation.

### 2.3. Diarrhetic shellfish toxins and standard materials

Okadaic acid (OA) (95%, Sigma, Gillingham, UK) and dinophysistoxin-1 (DTX-1) (Calbiochem-Novabiochem, Nottingham, UK) were purchased. OA was used as the reference standard in this study. A certified standard lyophilised mussel material containing diarrhetic toxins (MUS-2, National Research Council, Halifax, Canada) was reconstituted in methanol immediately prior to use to give a mixture with 2.5 $\mu$g total toxins/ml (2.29 $\mu$g of OA

and 0.21 μg of DTX-1/ml). DTX-2 was isolated from contaminated mussels as outlined below.

### 2.4. Chemicals

9-Anthryldiazomethane (ADAM) was purchased from Serva Feinbiochemica (Heidleberg, Germany). 1-bromoacetylpyrene (BAP) was purchased from Aldrich (Gillingham, UK) or was synthesised from pyrene following the method of Spijker et al. [16]. BAP was purified before use by chromatography on silica (silica gel 60, 70–230 mesh, E. Merck, Darmstadt, Germany) with elution using hexane–dichloromethane (1:2, v/v), followed by recrystallisation from dichloromethane–cyclohexane to give pale yellow needles with a m.p. of 129–130°C. Pyrenacyl okadaate (Sigma) was used for SPE recovery testing and deoxycholic acid (Sigma) was used as an internal standard. Water, methanol, acetonitrile and chloroform (stabilised with amylene, 50 ppm) were of HPLC grade (Labscan, Dublin, Ireland).

### 2.5. Calibrations for diarrhetic toxin analysis

Daily calibrations were performed using OA standards that were subjected to the same derivatisation and SPE procedures as described for shellfish analysis and results were based on peak areas. Calibrations using both derivatisation methods were linear for solutions containing 0.05–0.25 μg of OA, which represents 5–25 ng on-column; ADAM method (OA, $r=0.999$; DTX-2, $r=0.998$), BAP method (OA, $r=0.996$; DTX-2, $r=0.996$). Concentrations of DTX-1 and DTX-2 are expressed as OA equivalents and typical calibration curves for DTX-2 are shown in Fig. 4. The relative standard deviation for the determination of OA in the reference material, MUS-2, was 8% ($n=25$), using the BAP derivatisation procedure.

### 2.6. Preparation of shellfish samples

A regular screening programme for diarrhetic shellfish toxins was used to identify mussels (*Mytilus edulis*) that were contaminated with DTX-2 [17]. The digestive glands (hepatopancreas) were cut from mussels collected from the south-west coast of Ireland and stored at −20°C prior to extraction. Extraction of toxins was carried out following a modified procedure of Lee et al. [8]. A portion of homogenised shellfish hepatopancreas equivalent to 6 g was extracted with methanol–water (4:1, v/v) (12 ml) and, after centrifugation at 3000 rpm (10 min), an aliquot (2.5 ml) of the supernatant was washed with petroleum spirit (40–60°C), 2×2.5 ml, by vortex-mixing for 1 min. The upper layer was discarded each time and water (1 ml) and chloroform (4 ml) were added to the residual solution which was vortex-mixed for 2 min. After centrifugation (5 min), the lower chloroform layer was transferred, using a pipette, to a volumetric flask (10 ml). The chloroform extraction was repeated and the extracts were combined and made up to 10 ml with chloroform. An aliquot (0.5 ml) of this chloroform extract was evaporated under nitrogen and used for derivatisation.

### 2.7. Derivatisation with 1-bromoacetylpyrene (BAP)

Shellfish extract or OA, DTX-1 and DTX-2 standards (0.050–0.250 μg) in acetonitrile (0.1 ml), deoxycholic acid (0.10 μg) (internal standard, in acetonitrile, 0.1 ml), BAP (0.1% w/v in acetonitrile, 0.5 ml) and diisopropylethylamine (0.04 ml, 5% in acetonitrile) were mixed, ultrasonicated for 5 min and heated at 75°C for 20 min, while protected from light. Solvent was removed under nitrogen and the residue was reconstituted in chloroform–hexane (50:50, v/v; 1 ml). This mixture was subjected to clean-up using silica solid phase extraction (SPE) (Supelclean LC-Si, 3 ml, Supelco, Poole, UK). Chloroform, stabilised with amylene, was used in this procedure and this was adjusted with ethanol to produce a concentration of 1.2% (v/v). The SPE cartridge was conditioned with chloroform–hexane (50:50, v/v; 3 ml) prior to application of the sample. After washing with the conditioning solvent (5 ml), followed by chloroform (5 ml), the toxin derivatives were eluted with chloroform–methanol (95:5, v/v; 5 ml). After evaporation to dryness under nitrogen (40°C, Turbo Vap LV, Zymark), the residue was reconstituted in methanol (200 μl). Analysis by HPLC using a 20-μl injection gave 5–25 ng of toxin standards on-column.

### 2.8. Derivatisation with 9-anthryldiazomethane (ADAM)

ADAM solution (0.2%) was prepared by dissolving ADAM (6 mg) in acetone (50 μl) which was made up to 3 ml with methanol and filtered (0.45 μm membrane) for immediate use. Standard toxins and sample extracts were evaporated under nitrogen and treated with ADAM solution (200 μl), ultrasonicated for 5 min and allowed to stand for 2 h. All solutions containing ADAM were protected from light. The SPE procedure was identical to that used in the BAP method.

### 2.9. Liquid chromatographic analysis

HPLC analysis of the BAP and ADAM derivatives of diarrhetic shellfish toxins was carried out using isocratic solvent mixtures of acetonitrile–methanol–water (see Fig. 3 and Figs. 5–7 for solvent compositions) with a flow-rate of 0.5 ml/min on an Ultremex $C_{18}$ column (5 μm, 250×3.2 mm, Phenomenex) at 30°C. Fluorimetric detection was used for both ADAM ($\lambda_{ex}$ 365 nm, $\lambda_{em}$ 412 nm) and BAP ($\lambda_{ex}$ 365 nm, $\lambda_{em}$ 418 nm) derivatives.

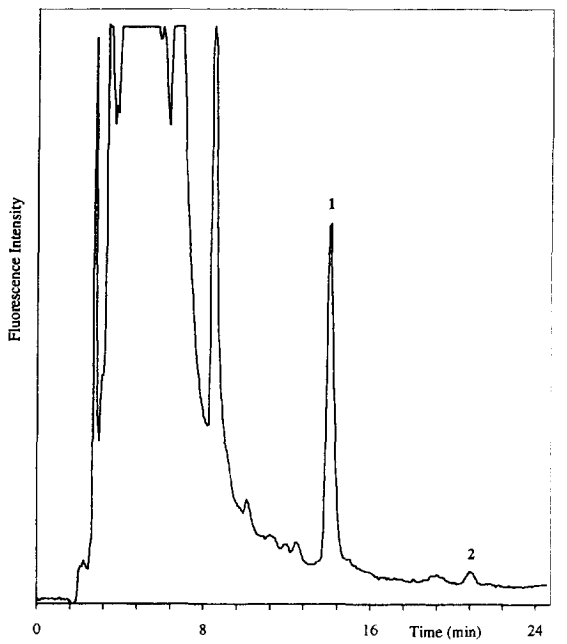


Fig. 3. Chromatogram of standard mussel material, MUS-2 (OA, 2.29 μg/ml; DTX-1, 0.21 μg/ml), extracted and derivatised using the BAP procedure (see Section 2). Peaks: 1, OA; 2, DTX-1. HPLC conditions: 5 μm Ultremex $C_{18}$ column (250×3.2 mm); eluent was acetonitrile–methanol–water (80:5:15, v/v).

### 2.10. Isolation of DTX-2 from shellfish

Mussels (*Mytilus edulis*) were collected from south-west Ireland following a bloom of phytoplankton (*Dinophysis acuta*). The hepatopancreas (550 g) were homogenised with methanol–water (80:20 v/v; 500 ml) for 15 min. The supernatant was washed with light petroleum (40–60°C, 2×500 ml) and extracted with chloroform (2×500 ml). The chloroform extracts were combined, dried ($MgSO_4$) and evaporated. In the following chromatographic steps, the fractions containing DTX-2 were identified by the analysis of aliquots by HPLC following derivatisation with ADAM or BAP:

(1) The residue was chromatographed on silica, 100 g (E. Merck) and eluted with ether followed by a step gradient of methanol–ether. DTX-2 eluted in fractions with 5–20% methanol–ether and this chromatography step was repeated.

(2) Fractions containing DTX-2 were transferred, using methanol, to a column containing Sephadex LH-20, 7 g (Pharmacia LKB, Uppsala, Sweden). DTX-2 eluted in methanol (20 ml) which was evaporated.

(3) An SPE cartridge, Mega Bond-Elut, $C_{18}$, 10 g (Varian, Harbor City, CA, USA), was conditioned with acetonitrile–water (40:60, v/v; 30 ml) and the DTX-2 fraction, in the same solvent, was applied to the cartridge. The SPE column was washed with acetonitrile–water (40:60, v/v, 30 ml and 60:40, v/v, 30 ml) and DTX-2 was eluted using acetonitrile–water (80:20. v/v; 30 ml).

(4) Semi-preparative HPLC was carried out using an Ultremex $C_{18}$ column (250×10 mm, 5 μm, Phenomenex). The mobile phase used was initially acetonitrile–water (50:50, v/v) for 5 min, increasing stepwise to 100% acetonitrile over 40 min and with a solvent flow of 4 ml/min. DTX-2 eluted mainly in acetonitrile–water (70:30, v/v) and other fractions containing less pure DTX-2 were re-chromatographed.

## 3. Results and discussion

### 3.1. Optimisation of derivatisation conditions for BAP

The derivatisation procedure using BAP was similar to that developed by Dickey et al. [11] for the determination of OA in shellfish and phytoplankton. A modified procedure has now been applied to the simultaneous analysis of the diarrhetic toxins, OA, DTX-1 and DTX-2 in shellfish. A number of factors were examined during the optimisation studies of this derivatisation, including temperature, reaction time and base concentration. Thus, using reaction temperatures for derivatisation ranging from 40–90°C, it was found that although peak areas increased with temperature, artefact peaks from reagent decomposition were observed above 75°C. At this temperature, maximum peak areas were observed after 15 min and remained unchanged for up to 30 min. The time selected for this protocol was 20 min. Improved derivatisation, resulting from increased base concentration, was also observed and 5% diisopropylethylamine was found to be optimum, a considerably higher concentration than that used previously [11].

### 3.2. Recovery studies for OA using the BAP procedure

The first part of this study examined the recoveries during the SPE stage, using pyrenacyl okadaate (BAP–OA), the expected product from the derivatisation reaction of OA with BAP. A sample of mussel hepatopancreas (non-toxic) was subjected to the usual extraction procedure and aliquots (10×0.5 ml) of the chloroform extract were spiked with BAP–OA (0.15 μg). After evaporation, five aliquots were subjected to the SPE procedure and the remaining were made up in methanol (200 μl) but were not subjected to SPE. Using 20-μl injection volumes, a comparison of the BAP–OA chromatographic peak areas for the two sets of samples showed that the recovery using SPE was 95±5%. Quilliam [9] has examined the SPE recoveries of the 9-anthrylmethyl derivatives and showed that inconsistent performance was related to a variable ethanol content in chloroform. Similar variability in the SPE of pyrenacyl

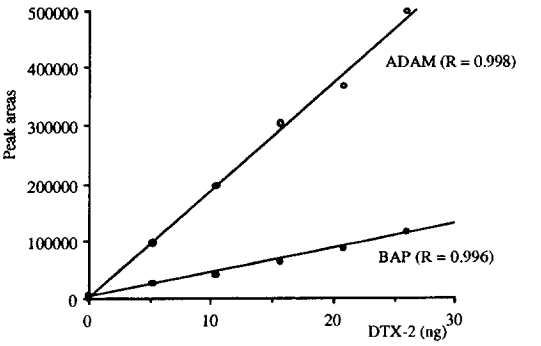


Fig. 4. Calibration curves for standard DTX-2 using ADAM and BAP derivatising reagents.

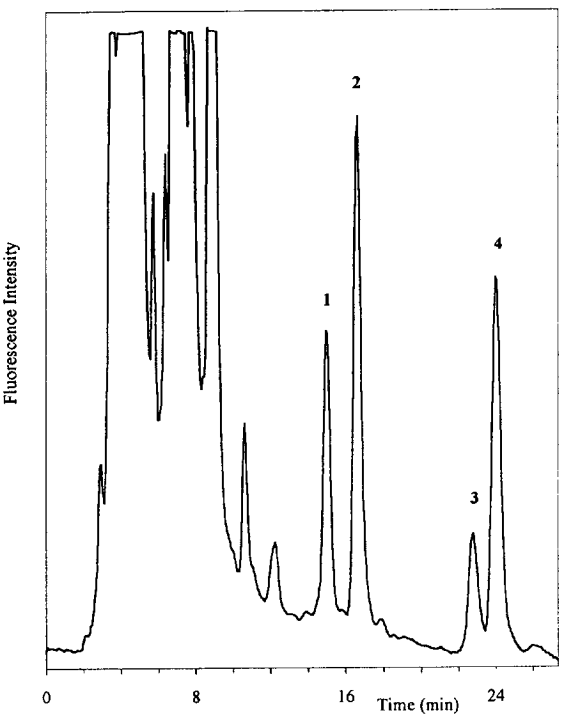


Fig. 5. Chromatogram of three diarrhetic shellfish toxin standards, derivatised using BAP. Peaks: 1, OA (15.2 min, 8 ng); 2, DTX-2 (16.8 min, 13 ng); 3, DTX-1 (23.1 min, 4 ng) and 4, deoxycholic acid (24.3 min, 10 ng, internal sandard). HPLC conditions: 5 μm Ultremex $C_{18}$ column (250×3.2 mm); eluent was acetonitrile–methanol–water (80:5:15, v/v).

derivatives was overcome by using ethanol-free chloroform (stabilised with amylene) and adjusting the ethanol content to an optimised value of 1.2% (v/v) before use.

To determine the overall recovery of the BAP analytical procedure, the mussel homogenate containing a certified OA content (MUS-2) was repeatedly analysed ($n=25$) and compared with the standard OA ($n=22$). The amount of OA to be injected was equivalent to 15 ng and the OA content in MUS-2 was $1.97\pm0.16$ $\mu$g/ml, which was 86% of the target value (2.29 $\mu$g/ml). A typical chromatogram from the analysis of MUS-2 is shown in Fig. 3.

### *3.3. Comparison of BAP and ADAM derivatisation methods*

Calibrations using DTX-2, isolated from shellfish, were linear for solutions containing 0.05–0.25 $\mu$g of DTX-2, which represents 5–25 ng on-column. The fluorescence response using ADAM was approximately four times greater than with BAP for DTX-2 (Fig. 4) and other toxins. However, one problem with ADAM is its instability and the quality of this reagent can vary considerably, resulting in artefact peaks in HPLC. In contrast, the chromatograms obtained using BAP were consistently good as shown in Fig. 5 which was obtained for a mixture of OA, DTX-1 and DTX-2 standards with deoxycholic acid (internal standard). The average detection limit for these toxins was 0.4 ng (on-column) using this reagent. Chromatograms with reagent blanks exhibited clean baselines in the region (10–20 min) where toxin analytes eluted (see insert, Fig. 7). Therefore, BAP was the preferred reagent for determining the purity of the diarrhetic shellfish toxins used as analytical standards.

### *3.4. Liquid chromatography of toxin derivatives*

The amount of toxin injected was 1–25 ng and, using the protocol for shellfish analysis outlined in Section 2, this is equivalent to a toxin content of 4.17 mg of mussel hepatopancreas. Typical chromatograms from the analysis of contaminated mussels are shown in Fig. 6. Using ADAM derivatisation to examine a mussel specimen collected in 1994, both OA and DTX-2 were present, with the latter toxin

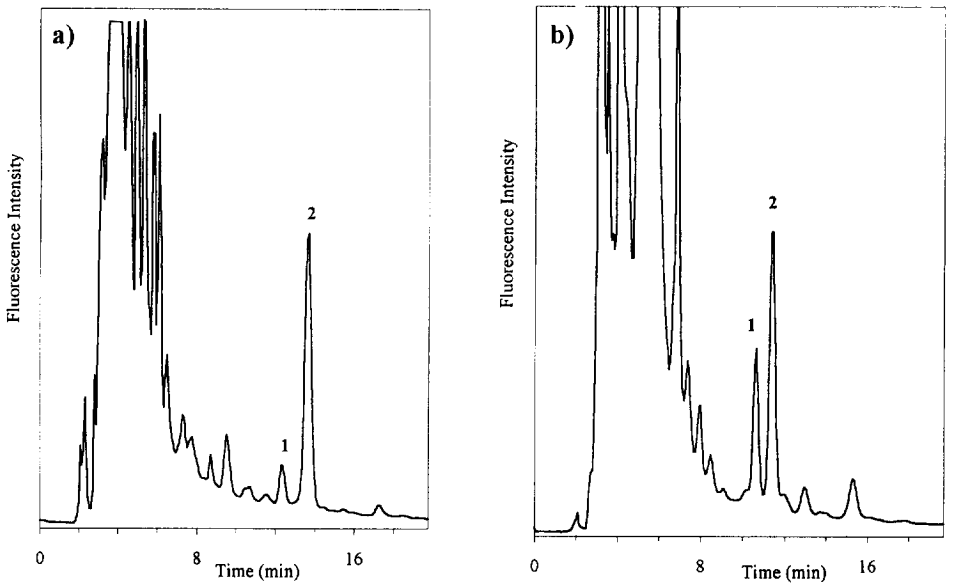


Fig. 6. Sample chromatograms from contaminated mussels obtained using (a) ADAM derivatisation method; (1) OA (12.5 min, 1.2 ng) and (2) DTX-2 (13.8 min, 10 ng). (b) BAP derivatisation method; (1) OA (10.7 min, 6.2 ng) and (2) DTX-2 (11.6 min, 11.3 ng). HPLC conditions: 5 $\mu$m Ultremex $C_{18}$ column (250×3.2 mm); eluents were acetonitrile–methanol–water, (a) 80:8:12 (v/v) and (b) 80:10:10 (v/v). Shimadzu RF551 detector gain settings were (a) 4 and (b) 16.

predominating. Acetonitrile–methanol–water (80:8:12, v/v) separated these isomeric toxins (Fig. 6a) and the retention times were 12.5 min (1.2 ng of OA) and 13.8 min (10 ng of DTX-2). A mussel sample, collected in 1995, was extracted and derivatised with BAP to give the chromatogram shown in Fig. 6b. The pyrenacyl derivatives of OA and DTX-2 were separated using acetonitrile–methanol–water (80:10:10, v/v) and the retention times were 10.7 min (6.2 ng, OA) and 11.6 min (11.3 ng, DTX-2). The anthrylmethyl and pyrenacyl derivatives of these diarrhetic toxins exhibited similar chromatographic behaviour and a useful application is the confirmation of toxin identity, based on retention times of two different derivatives.

### 3.5. Isolation of DTX-2 from mussels

An extensive programme involving the weekly examination of cultivated mussels (*Mytilus edulis*) from south-west Ireland for diarrhetic toxins allowed the acquisition of sample material containing DTX-2 [17]. HPLC analysis, following derivatisation with ADAM, revealed DTX-2 as the predominant toxin, with small amounts of OA. Bivalve shellfish, including mussels, scallops and clams, accumulate diarrhetic toxins in their digestive glands (hepatopancreas) after feeding on certain toxigenic algae [18]. The isolation of diarrhetic toxins from shellfish is difficult, due to the low natural abundance of these compounds, typically less than 1 $\mu$g/g of shellfish meat. Also, the lack of a suitable chromophore requires the extensive analysis of eluates collected, using either of the HPLC methods described in Section 2.

Only the hepatopancreas of mussels (20–25% of total meat) was used for toxin isolation. After homogenisation with methanol, extraction with light petroleum removed most lipids and acylated toxins (Fig. 1, $R_1$=acyl). OA and DTX-2 were extracted into chloroform and multiple chromatographic steps were required to purify the lipophylic toxin, DTX-2 (see Section 2). DTX-2, isolated from mussels, was 95% pure using HPLC analysis of both its 9-anthrylmethyl and pyrenacyl (Fig. 7) derivatives. Samples of DTX-2 have been supplied to a number of European Biotoxin Reference Laboratories for use as an analytical standard. Also, an ELISA test (DSP-Check, Sceti, Tokyo, Japan), which was developed to determine OA, was recently evaluated and this showed a 40±5% cross-reactivity with this standard DTX-2 [19].

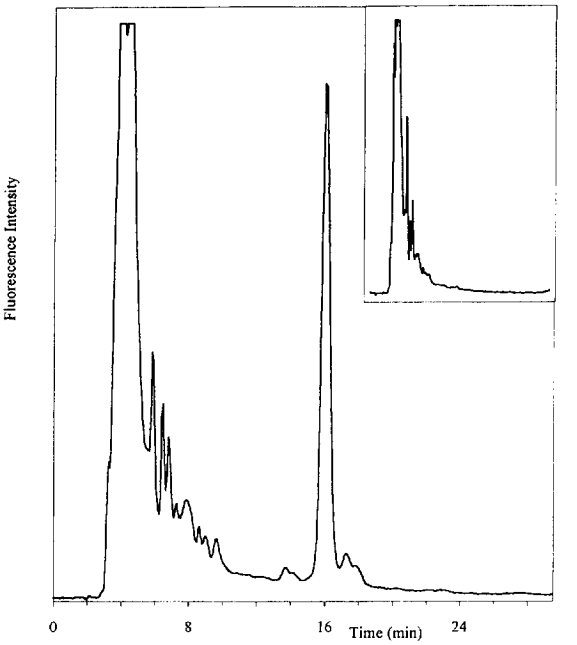


Fig. 7. Chromatogram of DTX-2, isolated from mussels, after derivatisation using BAP. Inset shows a typical reagent blank chromatogram. HPLC conditions: 5 $\mu$m Ultremex $C_{18}$ column (250×3.2 mm); eluent was acetonitrile–methanol–water (80:5:15, v/v).

## Acknowledgments

Financial support for this research (AR 013/95) and a studentship award (to S.S.K.) from Forbairt, the State Agency in Ireland for Science and Technology, are gratefully acknowledged. We also thank Pepsi Cola (Ireland) for the donation of HPLC equipment.

## References

[1] J.S. Lee, T. Igarashi, S. Fraga, E. Dahl, P. Hovgaard and T. Yasumoto, J. Appl. Phycol., 1 (1989) 147.

[2] H.P. van Egmond, T. Aune, P. Lassus, G.J.A. Speijers and M. Waldock, J. Nat. Toxins, 2 (1993) 41.
[3] P. Cohen, C.F.B. Holmes and Y. Tsukitani, Trends Biochem. Sci., 15 (1990) 98.
[4] T. Hu, J. Doyle, D. Jackson, J. Marr, E. Nixon, S. Pleasance, M.A. Quilliam, J.A. Water and J.L.C. Wright, J. Chem. Soc. Chem. Commun., 30 (1992) 39.
[5] E.P. Carmody, K.J. James and S.S. Kelly, Toxicon, 34 (1996) 351.
[6] J. Blanco, M. Fernandez, J. Marino, B. Reguera, A. Miguez, J. Maneiro, E. Cacho and A. Martinez, in P. Lassus, G. Arzul, E. Erard, P. Gentien and C. Marcaillou (Editors), Harmful Marine Algal Blooms, Lavoisier Science Publishers, Paris, 1995, p. 777.
[7] P. Vale and M.A. Sampayo, VIIth International Conference on Toxic Marine Phytoplankton, Sendai, Japan, 1995.
[8] J.S. Lee, T. Yanagi, R. Kenma and T. Yasumoto, Agric. Biol. Chem., 51 (1987) 877.
[9] M.A. Quilliam, J. AOAC Int., 78 (1995) 555.
[10] S. Allenmark, M. Chelminska-Bertilsson and R.A. Thomson, Anal. Biochem., 185 (1990) 279.
[11] R.W. Dickey, H.R. Granade and F.A. Bencsath, in T.J. Smayda and Y. Shimizu (Editors), Toxic Phytoplankton Blooms in the Sea, Elsevier, Newport, RI, 1993, p. 495.
[12] S.L. Morton and J.W. Bomber, J. Appl. Phycol., 6 (1994) 41.
[13] C. Hummert, B. Luckas and J. Kirschbaum, in P. Lassus, G. Arzul, E. Erard, P. Gentien and C. Marcaillou (Editors), Harmful Marine Algal Blooms, Lavoisier Science Publishers, Paris, 1995, p. 297.
[14] H. Ohrui, K. Akasaka, H. Meguro and T. Yasumoto, 19th International Symposium on Column Liquid Chromatography (HPLC'95), Innsbruck, Austria, 1995.
[15] B. Luckas, J. Chromatogr., 624 (1992) 439.
[16] N.M. Spijker, A.M. van den Bracken-van Leersum, J. Lugtenburg and J. Cornelisse, J. Org. Chem., 55 (1990) 757.
[17] E.P. Carmody, K.J. James, S.S. Kelly and K. Thomas, in P. Lassus, G. Arzul, E. Erard, P. Gentien and C. Marcaillou (Editors), Harmful Marine Algal Blooms, Lavoisier Science Publishers, Paris, 1995, p. 273.
[18] M. Murata, M. Shimatani, H. Sugitani, Y. Oshima and T. Yasumoto, Bull. Jpn. Soc. Sci. Fish., 48 (1982) 549.
[19] E.P. Carmody, K.J. James and S.S. Kelly, J. AOAC Int., 78 (1995) 1403.