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Liquid chromatography with fluorimetric, mass spectrometric and tandem mass spectrometric detection for the investigation of the seafood-toxin-producing phytoplankton, *Dinophysis acuta*

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Abstract

The diarrhoeic shellfish poisoning (DSP) toxins, okadaic acid (OA) and its isomer, dinophysistoxin-2 (DTX-2), were determined in the marine phytoplankton, *Dinophysis acuta*, harvested in Ireland. Unialgal samples (22–100 cells) were extracted and derivatised using 9-anthryldiazomethane (ADAM) or 1-bromoacetylpyrene (BAP) and analysed by liquid chromatography (LC). Isocratic elution on a C_{18} reversed-phase column, with fluorimetric detection, was used to determine OA (58±7 pg/cell) and DTX-2 (78±14 pg/cell). The detection limit was 0.1 ng OA/20 μl injection using ADAM. Gradient LC, using a polymeric bonded phase, successfully separated mixtures containing both the ADAM and BAP derivatised toxins. Identification of DSP toxins was confirmed using isocratic micro LC with tandem mass spectrometric (μLC–MS–MS) analysis of the free toxins and μLC–MS of the BAP-derivatised toxins with an ionspray (IS) interface, coupled to an atmospheric pressure ionisation (API) source. Collision induced dissociation (CID) ion mass spectra of the protonated molecule, $[M+H]^+$, at m/z 805 for OA and DTX-2, identified three diagnostic fragment ions for each analyte which were used for selected reaction monitoring (SRM) LC–MS–MS analysis. The detection limit for OA and DTX-2 was 0.025 ng/0.2 μl injected. These studies showed that *D. acuta* was the progenitor of DTX-2 in shellfish. © 1997 Elsevier Science B.V.

Keywords: Diarrhetic shellfish poisoning; Derivatization, LC; *Dinophysis acuta*; Toxins; Okadaic acid; Dinophysistoxin-2

1. Introduction

Diarrhoeic shellfish poisoning (DSP) is a severe gastrointestinal disturbance that results from eating contaminated shellfish [1]. The illness is produced by toxins that accumulate in bivalve shellfish, especially mussels, clams and scallops. It has been demonstrated that DSP toxins in shellfish originate from toxigenic phytoplankton (*Dinophysis* and *Prorocentrum* spp.) that shellfish have ingested [2,3]. Although pectenotoxins [4] and yessotoxins [5] have been associated with incidences of DSP, the okadaic acid (OA) group of toxins have been responsible for most outbreaks of this illness [6]. Dinophysistoxin-1 (DTX-1) and OA are found throughout the world with the latter being the most common DSP toxin in Europe [7]. However, the rare toxin, dinophysistoxin-2 (DTX-2), which is an isomer of OA (Fig. 1), was recently isolated from mussels (*Mytilus edulis*) [8,9]. This was shown to be the predominant DSP toxin in Ireland [10] and it has also been found in Spain [11] and Portugal [12].

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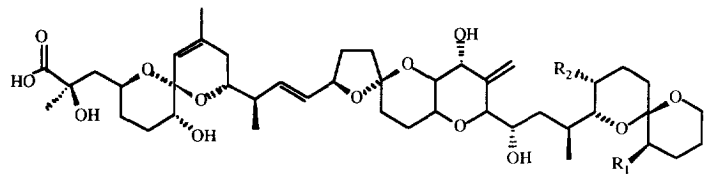

Fig. 1. Structures of diarrheoic shellfish toxins. Okadaic acid (OA) R_1=H, R_2=CH_3; dinophysistoxin-1 (DTX-1) R_1=CH_3, R_2=CH_3; dinophysistoxin-2 (DTX-2) R_1=CH_3, R_2=H.

The determination of DSP toxin profiles in shellfish and phytoplankton requires sensitive liquid chromatographic (LC) methods with fluorimetric or mass spectrometric detection. A number of fluorimetric derivatisation reagents have been used for the analysis of DSP toxins including, N-(9-acridinyl)-bromoacetamide [13], 4-bromomethyl-7-methoxycoumarin (Br-Mmc) [14] and 2,3-(anthracenedicarboximido)ethyltrifluoro - methanesulphonate (AE-OTf) [15]. However, the polyaromatic hydrocarbon reagents, 9-anthryldiazomethane (ADAM) [16,17], 1-pyrenyldiazomethane (PDAM) [18] and 1-bromoacetylpyrene (BAP) [9,19] have proved to be the most successful as they are less prone to interferences from reagent and reaction artefact compounds.

Liquid chromatography–mass spectrometry (LC–MS) has been shown to be a valuable analytical tool for confirming the identities of known toxins and identifying new compounds [17,20–24] in the toxin profiles of phytoplankton and shellfish. Micro liquid chromatography–tandem mass spectrometry (μLC–MS–MS) is a particularly useful method for handling very small samples with low analyte concentrations. This was particularly useful in this study since the only method of obtaining a unialgal sample of *D. acuta* was to pick individual cells from a microscope slide. Toxin studies on marine phytoplankton of the *Dinophysis* spp. are difficult as they cannot be cultured in the laboratory and wild populations are always mixed with other phytoplankton species [25]. In this paper, we report the application of sensitive LC methods to unambiguously identify and quantify DSP toxins in *Dinophysis acuta* and, for the first time, to show that DTX-2 is produced by this dinoflagellate.

2. Experimental

2.1. Reagents

9-Anthryldiazomethane (ADAM, Serva Feinbiochemica, Heidelberg, Germany) and 1-bromoacetylpyrene (BAP, Aldrich, Gillingham, UK) were purchased. All solvents were LC grade and were purchased from Farmitalia Carlo Erba (Milan, Italy) or Labscan (Dublin, Ireland). Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA). OA was purchased from Boehringer (Mannheim, Germany) and a certified reference standard solution (25.3 μg OA/ml) was obtained from the National Research Council (Halifax, Canada). DTX-2 was isolated from naturally contaminated mussels as described elsewhere [9]. Individual standard stock solutions containing 5 μg/ml of OA and DTX-2 were obtained from the pure compounds by dilution with methanol. Working solutions were obtained from standard stock solutions by appropriate dilutions with methanol.

2.2. Sample collection and preparation

The phytoplankton samples were collected from the subsurface (5–10 m depth) in the coastal areas of Union Hall, County Cork, Ireland in August 1996, using a large 50/108 μm plankton net. Phytoplankton samples were preserved using dilute acetic acid, observed under a microscope (magnification ×40) and counted by the Uthermohl's method [26]. The natural phytoplankton community was dominated by *D. acuta* (70%) although other planktonic organisms were also found, including *Ceratium fusus* (16%) and *Protoperidinium* spp. (6%). A 1 ml sample of

this phytoplankton contained $2–12 \cdot 10^3$ cells of *D. acuta* and, after freeze–thawing to disrupt cells, this was extracted with chloroform (2×3 ml) and the volume was brought to 10 ml. An aliquot (100 μl) was taken for derivatisation using ADAM or BAP. Individual cells (20 batches of 100) of *D. acuta* were collected from the microscope slides using a micropipette within 48 h of harvesting. The DSP toxins were extracted from the collected cells (100) by freeze–thawing and using chloroform (2×0.5 ml) with sonication. The combined extracts were filtered (0.45 μm), evaporated under nitrogen, and dissolved in methanol (100 μl). This solution was used for derivatisation with ADAM or BAP and for μLC–MS–MS analysis.

2.3. Fluorimetric LC

The LC system consisted of either an LC-10AD pump (Shimadzu, Duisberg, Germany) or a Waters 600E System (Millipore, Milford, MA, USA), column oven (CTO-10A, Shimadzu), fluorescence detector (RF-551, Shimadzu) with an autosampler (ISS-100, Perkin-Elmer, Uberlingen, Germany). Isocratic LC, flow 0.5 ml/min, using CH_3CN–CH_3OH–water (80:10:10 or 80:5:15) was performed with a Prodigy C_{18} column (250×3.2 mm, 5 μm, Phenomenex, Macclesfield, UK), a precolumn (Prodigy C_{18}, 30×3.2 mm, 5 μm) and an in-line filter (0.5 μm×3 mm, Rheodyne, Cotati, CA, USA). Chromatographic data handling was performed using Unipac Class-VP software (Shimadzu) and data were transferred to Microsoft Excel for graphical presentation. Gradient LC was performed using an Envirosep-PP column (250×3.2 mm, Phenomenex) with a linear gradient of CH_3CN–water (54–71% CH_3CN, 61.5 min), at a flow of 1.5 ml/min. This gradient was optimised using DryLab software (LC Resources, Walnut Creek, CA, USA). Fluorimetric detection was used for both ADAM (λ_{ex} 365 nm, λ_{em} 412 nm) and BAP (λ_{ex} 365 nm, λ_{em} 418 nm) derivatives.

2.4. Derivatisation with 1-bromoacetylpyrene (BAP)

Using acetonitrile as the solvent for all solutions, phytoplankton extract or OA and DTX-2 standards (0.25–1.25 μg, 100 μl), BAP (0.1%, w/v, 500 μl) and diisopropylethylamine (5%, 40 μl) were mixed, sonicated for 5 min and heated at 75°C for 20 min, protected from light. Solvent was removed under nitrogen and the residue was reconstituted in chloroform–hexane (50:50, 1 ml) for clean-up using silica solid-phase extraction (SPE) as previously described [27]. After evaporation to dryness, under nitrogen, the residue was reconstituted in methanol (200 μl) and 20 μl was injected.

2.5. Derivatisation using 9-anthryldiazomethane (ADAM)

Derivatisations using ADAM were carried out using a modification [9] of the method developed by Lee et al. [16]. Standard toxins or phytoplankton extracts in methanol (100 μl) were evaporated under nitrogen and treated with ADAM solution (0.2% in methanol, 200 μl), sonicated for 5 min and allowed to stand for 2 h. All solutions containing ADAM were protected from light and the same SPE procedure was used as in the BAP method.

2.6. μLC–MS and μLC–MS–MS

Analyses were performed on a Phoenix 20 CU LC pump (Fison, Milan, Italy) liquid chromatograph. A Valco (Houston, TX, USA) injection valve, equipped with a 0.2 μl internal loop, was used for the injection of samples. Separation of toxins was carried out on a microcolumn packed with Supelcosil LC18-DB (Bellefonte, PA, USA) (300×1 mm, 5 μm) at room temperature, under isocratic conditions, with a mobile phase of acetonitrile–water (85:15, v/v) containing 0.1% TFA and a flow-rate of 30 μl/min.

Mass spectral analysis was performed on a PE-Sciex API III (PE-Sciex, Thornhill, Canada) triple quadrupole. The mass spectrometer was equipped with an atmospheric pressure ionisation (API) source and an ionspray interface set at a voltage of 5500 V. Ultra high purity (UHP) nitrogen was used as the curtain gas and nebulizer gas in the ionspray interface. Orifice potential voltage (OR) was set at 50 V. Full-scan mass spectra were acquired in single MS positive-ion mode both in flow injection analysis

(FIA) MS and in LC–MS experiments over the mass range m/z 700–1500. Data acquisition for μLC–MS analysis of OA and DTX-2, after derivatisation with BAP, was performed by selected ion monitoring (SIM) on the m/z 805 ion, corresponding to the protonated molecules, $[M+H]^+$ of OA and DTX-2 and on the m/z 1048 ion, corresponding to the protonated molecules, $[M+H]^+$ of BAP-OA and BAP-DTX2.

Analyses of underivatised algal extract samples were performed by μLC–MS–MS using selected reaction monitoring (SRM). The mass spectrometer was programmed to transmit the protonated molecule, $[M+H]^+$, through the first quadrupole (Q1) at m/z 805 both for OA and DTX-2. Following collision induced fragmentation in Q2 (collision gas argon) product ions were selected by Q3 at m/z value of 733, 751 and 769 both for OA and DTX-2. A collision energy of 20 eV was used.

The m/z values indicated both in text and in figures are in all cases the truncated values of the more accurate experimental values.

3. Results and discussion

There is circumstantial evidence that *Dinophysis acuta* is the progenitor of the rare diarrhoeic shellfish toxin, DTX-2, since the presence of this toxin in shellfish in south-west Ireland was observed soon after blooms of *D. acuta* in 1991 and 1994 [10]. Sensitive analytical methods are required to determine DSP toxins in phytoplankton and shellfish since these toxins have recently been shown to be potent tumour promoters at sub-acute levels [28]. Improved LC methods, with fluorimetric and MS detection, were therefore developed for the analysis of DSP toxins in phytoplankton and these methods were applied to establish the presence of DTX-2 in wild samples of *D. acuta*.

3.1. Determination of DSP toxins in phytoplankton using fluorimetric LC (ADAM method)

The presence of DSP toxins in small numbers of these cells was established using reversed-phase (RP) fluorimetric LC with the ADAM derivatisation method. Both OA and DTX-2 were identified by comparison of retention times with standard toxins but other potentially interfering peaks were also observed. Spiking experiments were therefore conducted to distinguish OA and DTX-2 from other closely eluting components in the phytoplankton. Fig. 2 shows the chromatograms for a sample extract from ca. 125 *D. acuta* cells and an equivalent sample that was spiked with OA (10 ng) and DTX-2 (10 ng). The symmetric increase in peak size for OA and for DTX-2 represents a 1 ng spike of each toxin per injection.

The natural phytoplankton sample was not a monoculture and contained 70% *D. acuta* along with other phytoplankton species. Since *Dinophysis* spp. cannot be cultivated in the laboratory, the determination of toxins in monocultures requires individual cells to be collected by picking them individually from a microscope slide [3]. The size of a typical *D. acuta* cell is 55–75 μm and the laborious collection procedure means that the total amount of unialgal sample available for analysis is limited. However, the sensitivity of this ADAM method can be appreciated from Fig. 3 which shows OA and DTX-2 in a chromatogram obtained from a sample of only 22 cells. The average amount per cell of these DSP toxins in *D. acuta*, determined on unialgal samples of 100 cells ($n=6$), was OA (58±7 pg) and DTX-2 (78±14 pg). DTX-1 was not detected in these samples.

3.2. μLC–MS and μLC–MS–MS analysis of underivatised DSP toxins

Ionspray MS has been shown in previous studies [17,23,24,29] to be a valuable technique for the determination of DSP toxins. Full-scan mass spectra, acquired in single MS positive ion mode, showed the protonated molecule $[M+H]^+$ at m/z 805 for OA and DTX-2 with no evidence of fragmentation [30]. Phytoplankton extracts were also analysed by μLC–MS in full scan mode (m/z 700–1500) and the total ion current traces only showed interesting peaks at m/z 805 (data not shown), suggesting the presence of OA and DTX-2 in these samples. The intact protonated molecule of each analyte served as the precursor ion for CID in the MS–MS experiments. The OA spectrum confirmed the fragmentation previously obtained [29] through LC–MS–MS experiments

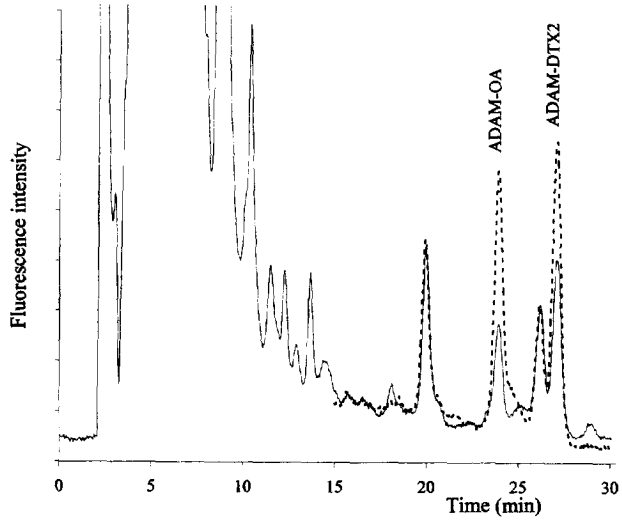

Fig. 2. Chromatogram from the fluorimetric LC analysis of a phytoplankton extract (0.1 ml, ca. 125 *D. acuta* cells), following derivatisation using ADAM. Overlay (- - -) is from an equivalent sample, spiked with OA (10 ng) and DTX-2 (10 ng). Final volume was 200 μl. Conditions: Prodigy C_{18} column (250×3.2 mm, 5 μm); mobile phase: acetonitrile–methanol–water (80:5:15); flow-rate 0.5 ml/min; injection volume 20 μl; detection: λ_{ex} 365 nm, λ_{em} 412 nm.

with an API source and an IS interface. DTX-2, an isomer of OA (Fig. 1), gave the same fragment ions as OA [31].

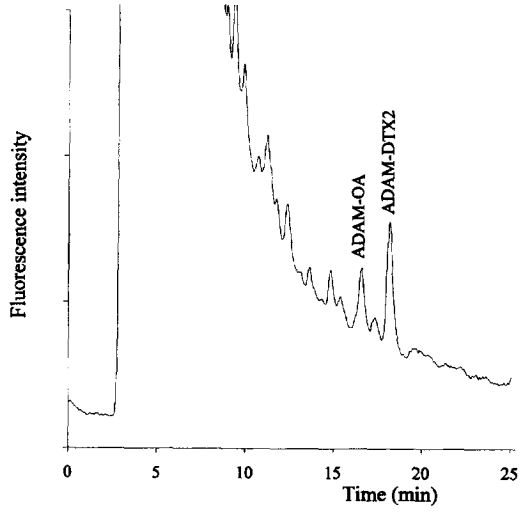

Fig. 3. Chromatogram from the fluorimetric LC analysis of a unialgal *D. acuta* extract (0.1 ml, 22 cells), following derivatisation using ADAM. Conditions: Prodigy C_{18} column (250× 3.2 mm, 5 μm); mobile phase: acetonitrile–methanol–water (80:10:10); flow-rate 0.5 ml/min; injection volume 20 μl; detection: λ_{ex} 365 nm, λ_{em} 412 nm.

The adoption of micro columns significantly facilitates the determination of small samples and/or low concentrations of analytes. Recently, we proposed an analytical procedure for determining DSP toxins in mussels using μLC–MS–MS [31]. In order to achieve targeted analyses and maximum sensitivity, the SRM μLC–MS–MS of the phytoplankton extracts was implemented by adopting a small volume injection valve (0.2 μl). A micro-LC18 column (1 mm I.D.), with an optimum binary mobile phase of aqueous acetonitrile with 0.1% TFA, was used which permitted low-flow LC–MS–MS to be carried out without column eluate splitting. Using a mixture of toxin standards, excellent signals and separations were achieved for OA and DTX-2 which eluted as sharp symmetrical peaks at 13 min and 15 min, respectively (Fig. 4A). The detection limit, based on a *S/N* ratio of at least 3:1, was estimated to be 0.025 ng (injected) for both OA and DTX-2.

Fig. 4B shows the SRM μLC–MS–MS analysis of a phytoplankton extract containing ca. $2 \cdot 10^3$ *D. acuta* cells per ml. The unambiguous identification of OA and DTX-2 in the sample was made based on retention time, molecular mass ($[M+H]^+$, m/z 805), structural information, such as the presence of three diagnostic fragments (m/z 805⇒751, m/z 805⇒769

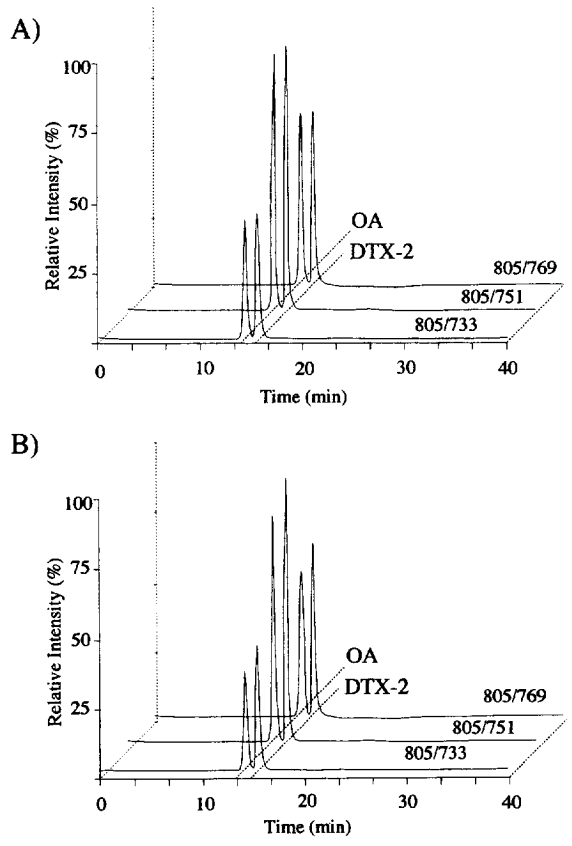

Fig. 4. SRM μLC–MS–MS chromatograms of (A) standard mixture containing 0.5 μg/ml of OA and DTX-2 and (B) methanol underivatised extracts of phytoplankton containing ca. $2 \cdot 10^3$ *D. acuta* cells per ml. Conditions: column: Supelcosil LC18-DB (300×1 mm, 5 μm); mobile phase: acetonitrile–water (85:15, v/v) containing 0.1% TFA; flow-rate of 30 μl/min; injection volume: 0.2 μl. SRM was implemented using the parent–daughter ion combinations of *m/z* 805⇒733, 805⇒751 and 805⇒769, both for OA and DTX-2; collision energy of 20 eV was used.

and *m/z* 805⇒733) for each analyte, and their ion ratios.

3.3. Fluorimetric LC analysis of BAP and ADAM-derivatised toxins

Calibrations using both the ADAM and BAP derivatisation methods were linear for solutions prepared from the certified okadaic acid standard; ADAM method (0.26–1.30 μg OA/ml, r=0.998), BAP method (0.52–2.6 μg OA/ml, r=0.996). The detection limits for OA (S/N 3:1) were 0.1 ng (ADAM) and 0.4 ng (BAP) for 20 μl injections. Although the ADAM method for the analysis of DSP toxins is more sensitive than the BAP method, the latter is preferred for determining toxin purity [9]. In

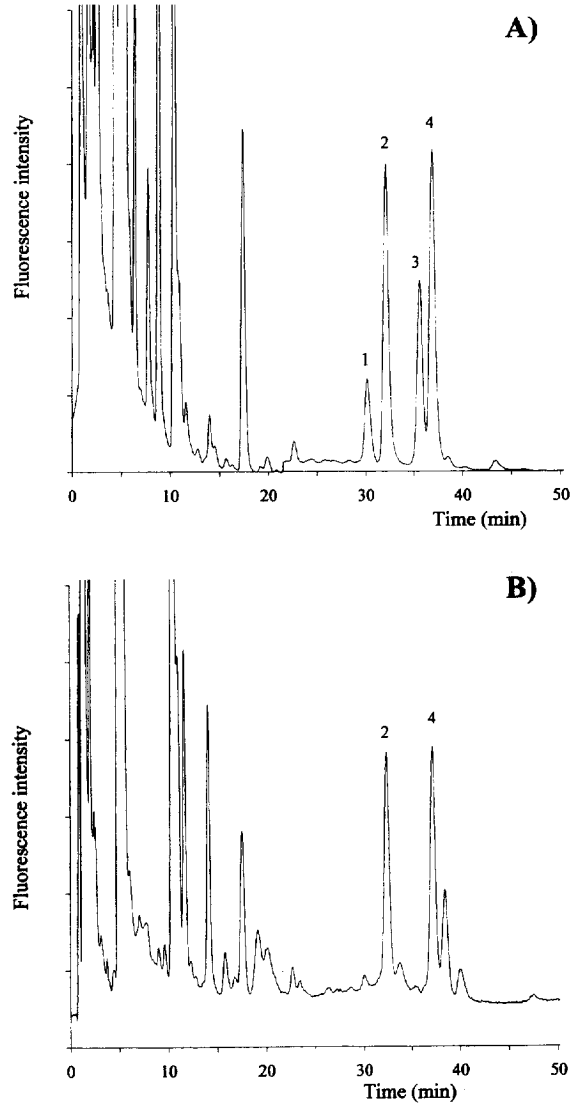

Fig. 5. (A) Fluorimetric LC of standard OA and DTX-2, separately derivatised using ADAM and BAP and mixed prior to injection. 1 ADAM-OA, 2 BAP-OA, 3 ADAM-DTX2, 4 BAP-DTX2. Detection: λ_{ex} 365 nm, λ_{em} 412 nm. (B) Fluorimetric LC analysis of a phytoplankton extract (0.1 ml, ca. 125 *D. acuta* cells), following derivatisation using BAP. Detection: λ_{ex} 365 nm, λ_{em} 418 nm. Conditions: Envirosep-PP column (250×3.2 mm); mobile phase: linear gradient of acetonitrile–water (54–71% acetonitrile, 61.5 min); flow-rate of 1.5 ml/min.

addition, there are advantages in establishing the identity of a toxin by examining the chromatographic behaviour of more than one derivative. Unfortunately, LC with a C_{18} column did not adequately separate the BAP and ADAM derivatives of OA and DTX-2. We therefore examined the possibility of improving the selectivity by using an LC column that was developed for the analysis of polyaromatic hydrocarbons since the critical separations were the 9-anthrylmethyl and pyrenacyl derivatives of each toxin. Using an optimised gradient of acetonitrile–water with a cross-linked polymeric column, Envirosep-PP, satisfactory separations of the four derivatisation products was achieved (Fig. 5A) with retention times, 30.07 min (ADAM-OA), 31.95 min (BAP-OA), 35.48 min (ADAM-DTX2) and 36.75 min (BAP-DTX2). A sample of the phytoplankton extract, derivatised with BAP is shown in Fig. 5B and the retention times for these derivatives were within 0.1% of those using standards.

3.4. μLC–MS analysis of DSP toxins, derivatised using BAP

A previous study [19] on the use of BAP for the derivatisation of OA confirmed the identity of the pyrenylokadaate peak, observed using fluorimetric LC, by the mass spectrum of its silylated derivative. The possibility of using μLC–MS to directly analyse BAP-derivatised phytoplankton extracts was examined to confirm the identities of the derivatised toxins, OA and DTX-2, detected by fluorimetric analysis. The full-scan single MS positive ionspray mass spectra (*m/z* 700–1500) are shown in Fig. 6A and B, respectively. These spectra were obtained from a 0.2 μl injection of 5 μg/ml solutions of toxins, derivatised using BAP, into a 30 μl/min flow of mobile phase using the FIA technique. These spectra are simple, exhibiting an abundant peak due to the protonated molecule, $[M+H]^+$, at *m/z* 1048, both for BAP-OA and BAP-DTX2. A peak due to the sodium adduct, $[M+Na]^+$, at *m/z* 1070, was also observed in the spectra of both BAP-derivatised toxins. The protonated molecules were considered suitable for SIM μLC–MS analyses of BAP-derivatised toxins.

The SIM μLC–MS chromatograms of a mixture of toxin standards, derivatised with BAP, are shown in Fig. 7A. The analytes were eluted at 29 min (BAP-OA) and 30 min (BAP-DTX2) and a detection limit of 0.055 ng/injection was estimated for the toxins, which is slightly higher than that obtained by

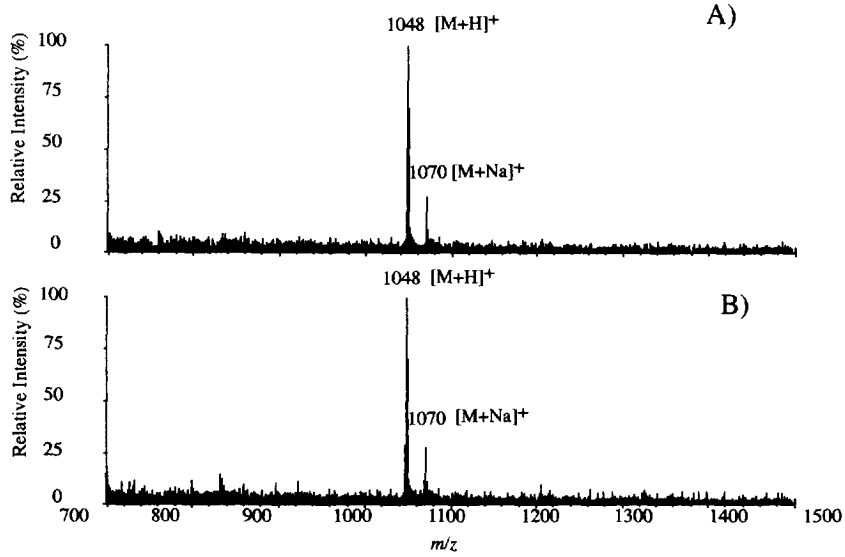

Fig. 6. Positive ionspray mass spectra of individual solutions containing 5 μg/ml of (A) OA derivatised with BAP and (B) DTX-2 derivatised with BAP. Conditions: flow injection analysis (FIA); mobile phase: acetonitrile–water (85:15, v/v) containing 0.1% TFA; flow-rate of 30 μl/min; injection volume: 0.2 μl.

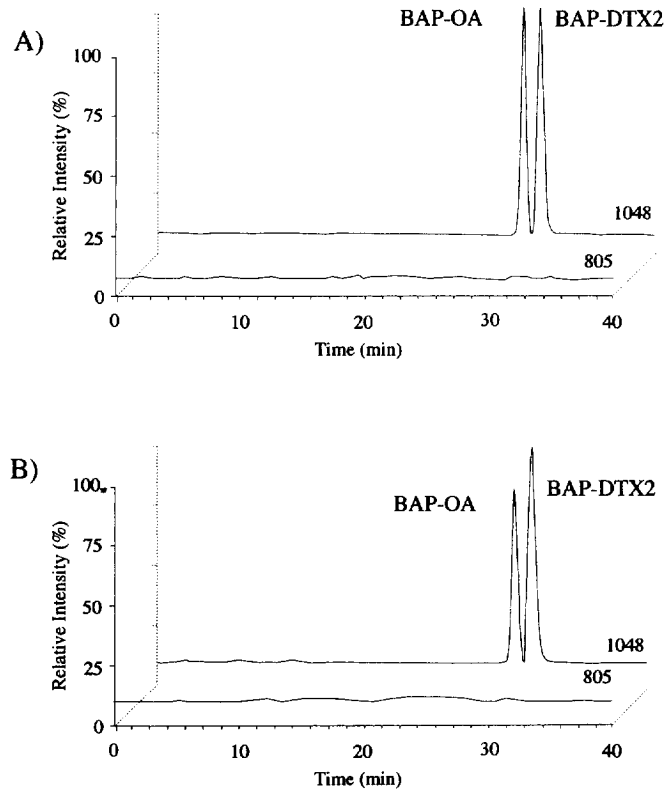

Fig. 7. SIM μLC–MS chromatograms of (A) standard mixture containing 0.5 μg/ml of BAP-derivatised OA and BAP-derivatised DTX-2 and (B) BAP-derivatised extract of phytoplankton containing ca. $2 \cdot 10^3$ *D. acuta* cells per ml. Conditions: column: Supelcosil LC18-DB (300×1 mm, 5 μm); mobile phase: acetonitrile–water (85:15, v/v) containing 0.1% TFA; flow-rate of 30 μl/min; injection volume: 0.2 μl. SIM on the ion *m/z* 805, corresponding to the protonated molecules, $[M+H]^+$, of OA and DTX-2 and on the ion *m/z* 1048, corresponding to the protonated molecules, $[M+H]^+$, of BAP-OA and BAP-DTX2.

the SIM μLC–MS analysis of underivatised toxins (i.e., 0.035 ng/injection). This is consistent with the data reported by Pleasance et al. [22] who found that the sensitivity was lower for ADAM-derivatised OA, than for underivatised OA, using ionspray SIM LC–MS. One possible explanation for this phenomenon is the preference of the ionspray process for polar molecules such as underivatised acidic toxins.

The application to BAP-derivatised phytoplankton extracts was undertaken and Fig. 7B shows the SIM μLC–MS analysis from a sample containing ca. $2 \cdot 10^3$ *D. acuta* cells per ml. The presence of BAP derivatives of the DSP toxins is clearly indicated by the presence of chromatographic peaks at the *m/z* 1048, at the same retention of BAP-OA and BAP-DTX2, whereas the absence of peaks at *m/z* 805 indicated that the toxins were completely converted to their ester derivatives.

This study has unambiguously established, for the first time, that the diarrhoeic shellfish toxin, DTX-2, is produced by the marine phytoplankton, *Dinophysis acuta*.

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