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Liquid chromatography–electrospray ionization mass spectrometry of the diarrhetic shellfish-poisoning toxins okadaic acid, dinophysistoxin-1 and pectenotoxin-6 in bivalves

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Abstract

Determination of diarrhetic shellfish-poisoning (DSP) toxins, okadaic acid (OA), dinophysistoxin-1 (DTX1) and pectenotoxin-6 (PTX6) was carried out by liquid chromatography (LC) followed by on-line atmospheric pressure electrospray ionization-mass spectrometric (ESI-MS) detection with a heated capillary interface. Mass spectra of authentic OA, DTX1 and PTX6 standards exhibited abundant $[M-H]^-$ at m/z 803, 817 and 887, respectively. Linearity of peak area obtained by selected-ion monitoring (SIM) for $[M-H]^-$ of each toxin was confirmed over a wide range of concentrations from 10 pg to 30 ng. LC–ESI-MS analysis of OA, DTX1 and PTX6 in scallops and mussels, collected at the same site (Mutsu Bay, Japan), was carried out. Scallops and mussels collected at the same site showed different toxin profiles. Although PTX6 was detected from scallops, it was not detected from mussels.

Keywords: Shellfish poisoning; Okadaic acid; Dinophysistoxin-1; Pectenotoxin-6

1. Introduction

Diarrhetic shellfish poisoning (DSP) is a severe gastrointestinal illness caused by the consumption of shellfish contaminated by DSP toxin-producing dinoflagellates [1]. Three different groups of toxins isolated from scallops are implicated in DSP: homologues of okadaic acid (OA) (Fig. 1A), macrolide toxins called pectenotoxins (PTXs) (Fig. 1B), and yessotoxin (YTX) [2]. Of these, the OA homologues are the most important, because of their potent diarrheagenicity [3,4] and tumor-promoting activity [5]. The PTXs are thought to be mildly diarrhetic [6] and highly hepatotoxic [3,6]. Recently a new PTX homologue was found in a dinoflagellate (a *Dinophysis acuta* strain) from Ireland and in mussels from New Zealand [7]. PTX6 is thought to be the oxidation product of dinoflagellate-produced PTX2 in bivalves [8–11]. YTX has been suggested to be much less hazardous to human health than other DSP toxins [12].

Electrospray ionization (ESI) techniques have been demonstrated to be well-suited for the liquid chromatography–mass spectrometry (LC–MS) analysis of DSP toxins [11,13–24]. Although ESI has been performed by a wide variety of interfaces [25], most of the LC–MS studies of DSP toxins have been carried out with pneumatically assisted ESI (ion-

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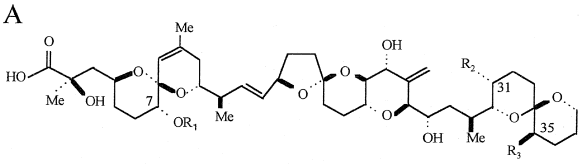
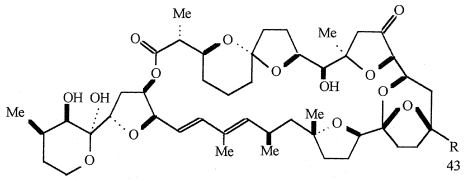
A



	R_1	R_2	R_3
okadaic acid (OA)	H	CH_3	H
dinophysistoxin-1 (DTX1)	H	CH_3	CH_3
dinophysistoxin-2 (DTX2)	H	H	CH_3
dinophysistoxin-3 (DTX3)	Acyl	CH_3	CH_3

B



R	
pectenotoxin-1 (PTX1)	CH_2OH
pectenotoxin-2 (PTX2)	CH_3
pectenotoxin-3 (PTX3)	CHO
pectenotoxin-6 (PTX6)	COOH

Fig. 1. Structures of typical OA (A) and PTX (B) derivatives. Molecular weights: OA=804, DTX1=818, PTX1=874, PTX2=858, PTX3=872, PTX6=888.

spray) involving droplet desolvation in a curtain gas flow. In the present study, LC–MS analysis of OA, DTX1 and PTX6 was carried out with a heated capillary ESI interface. As an application of the present LC–MS analysis, OA, DTX1 and PTX6 in scallops and mussels collected at the same site were determined and DSP toxin profiles were compared between bivalve species.

2. Experimental

2.1. Materials

Authentic OA, DTX1, and PTX6 were provided by Japan Food Research Laboratories (Tokyo, Japan) [26]. DTX2 was a generous gift from Dr. J.L.C. Wright [27]. Analytical-grade solvents (chloroform, dichloromethane, *n*-hexane, methanol), HPLC-grade solvents (acetonitrile, methanol) and analytical-grade reagents (acetic acid, HCl, NaOH) were purchased from Wako (Osaka, Japan). Distilled water was passed through a Milli-Q water purification system (Millipore, Bedford, MA, USA) and used for the mobile phase of LC–MS analysis.

2.2. Sample materials

Non-toxic scallops (*Patinopecten yessoensis*) and mussels (*Mytilus galloprovincialis*) were collected at Okatsu Bay, Japan, in May 1998. Toxic scallops (*P. yessoensis*) and mussels (*M. galloprovincialis*) were collected at the same site (within a radius of 3 m) at Mutsu Bay, Japan, in 1995. The specimens were subjected to the extraction of toxins, directly after the collection.

2.3. Extraction of DSP toxins from the midgut glands of bivalve samples

Extraction of OA, DTX1 and PTX6 from the midgut glands of bivalves was carried out according to a published method [28]. Combined midgut glands of five bivalves were extracted with four times the equivalent volume of methanol–water (8:2, v/v). After centrifugation, 2.5 ml of the supernatant corresponding to 500 mg of midgut glands of five bivalves was extracted twice with 2.5 ml of *n*-hexane to remove lipid components, then 1 ml of 0.2% acetic acid was added to the methanolic solution, and the toxins were extracted twice with 4 ml of chloroform. The combined chloroform extracts were made up to 10 ml with chloroform. A 1-ml aliquot of chloroform extract was put in a dark vial, and dried under nitrogen gas. The residue was re-dissolved in 100 μl of HPLC-grade methanol. A 2-μl aliquot was directly analyzed by LC–MS. The amount injected into the analytical system corresponded to the extract of 1 mg of midgut gland.

Tests of recovery of OA, DTX1 and PTX6 were carried out by LC–MS detection from the methanol–water (8:2, v/v) extracts obtained from the midgut glands (scallops and mussels) spiked with a known amount (0.2, 1.0 and 4.0 μg) of each toxin. The extraction procedures for toxins were as described above.

2.4. Hydrolysis of esterified DSP toxins

Aliquots (2.5 ml) of the methanol–water (8:2, v/v) extracts were extracted twice with *n*-hexane (2.5 ml) and these combined extracts were made up to 10 ml with *n*-hexane; it is known that 7-*O*-acyl DTX1 (that is: DTX3) preferably dissolves in *n*-hexane in this procedure [24,29]. A 1-ml aliquot of *n*-hexane extract was evaporated, then hydrolyzed in 100 μl of 0.5 N NaOH solution in methanol–water (9:1, v/v) at 75°C for 40 min. After evaporating the solvent from the reaction mixture, the residue was acidified with 300 μl of 0.5 N HCl, then extracted three times with 300 μl of diethyl ether. After evaporating the solvent, the extracts were dissolved in 250 μl of methanol–water (8:2, v/v) and then extracted twice with 250 μl of *n*-hexane to remove lipid components. One hundred μl of 0.2% acetic acid was added to the methanolic solution, then the resulting toxins were extracted twice with 400 μl of chloroform. The combined chloroform extract was evaporated under nitrogen gas, then dissolved in 100 μl of HPLC-grade methanol. A 2-μl aliquot was directly analyzed by LC–MS. The amount injected into LC–MS corresponded to the extract of 1 mg of midgut gland.

2.5. LC–MS

LC–MS was performed on a Hewlett–Packard Model 1050 Series liquid chromatograph coupled to a Finnigan MAT SSQ-7000 mass spectrometer (San Jose, CA, USA) equipped with an atmospheric pressure ESI interface and an ICIS data system. Separation of OA, DTX1 and PTX6 was achieved on a Mightysil RP-18 column containing octadecyl phase bonded to 5-μm silica gel particles (150×2 mm I.D.; Kanto, Tokyo, Japan) connected with a guard column Mightysil RP-18 (5×2 mm I.D.) at 35°C. HPLC-grade acetonitrile–water (7:3, v/v) containing 0.1% acetic acid was used as the mobile phase at a flow-rate of 200 μl/min. The LC flow was introduced into the ESI interface without any splitting. The spray capillary voltage on the ESI interface was maintained at approximately 4.5 kV. The skimmer voltage was 74 V. The temperature of the heated capillary was 200°C. High-purity nitrogen gas was used as a sheath gas (nebulizer gas) at an operating

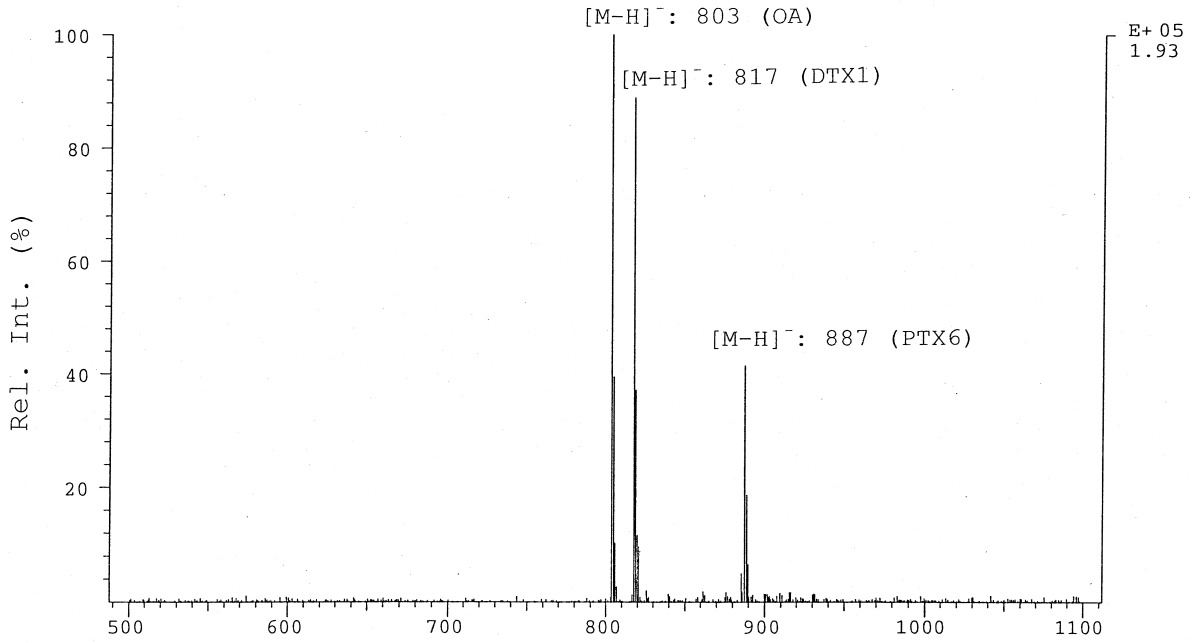


Fig. 2. Negative-ion mass spectra of a mixture of OA, DTX1 and PTX6 standards obtained by FIA into 200 μl/min flow of acetonitrile–water (7:3, v/v) with 0.1% acetic acid. A mixture of 2 ng of each toxin was injected.

pressure of 70 p.s.i. and an auxiliary gas at five units, respectively (1 p.s.i.=6894.76 Pa). Toxins were scanned with selective ion monitoring (SIM) of negatively charged ions at *m/z* of 803.5 (OA), 817.5 (DTX1) and 887.5 (PTX6) with a scan-time of 1.0 s. The SIM scan width for each toxin was 0.3 U (unit mass). The MS parameters were optimized for the ionization of standard toxins using flow injection analysis (FIA).

2.6. Column chromatography of the unknown compound with m/z 803 in the bivalve extracts

An aliquot of chloroform extract of the midgut glands of bivalves obtained after extraction from aqueous methanol was evaporated and diluted in dichloromethane–methanol (1:1, v/v), then loaded on a Sep-Pak plus alumina B cartridge (Waters, Milford, MA, USA) which had been previously conditioned with 10 ml of dichloromethane–methanol (1:1, v/v). The column was washed with 10 ml of dichloromethane–methanol (1:1, v/v) followed by 10 ml of methanol, and finally washed with 10 ml of 1% NH_4OH–methanol (1:1, v/v) [26,30]. Each fraction was collected, evaporated to dryness, and re-dissolved in 100 μl of methanol; 2 μl of the final solution was subjected to LC–MS analysis.

3. Results and discussion

3.1. FIA and LC–MS of OA, DTX1 and PTX6 standard toxins

Fig. 2 shows the full-scan negative-ion ESI mass spectrum of OA, DTX1 and PTX6 obtained by FIA. OA, DTX1 and PTX6 yielded mass spectra exhibiting abundant $[M\text{-}H]^-$ at *m/z* 803, 817 and 887, respectively. The efficiency of ionization with acetonitrile–water (7:3, v/v) containing 0.1% formic acid or 1 m*M* ammonium acetate was inferior to that obtained in acetonitrile–water (7:3, v/v) containing

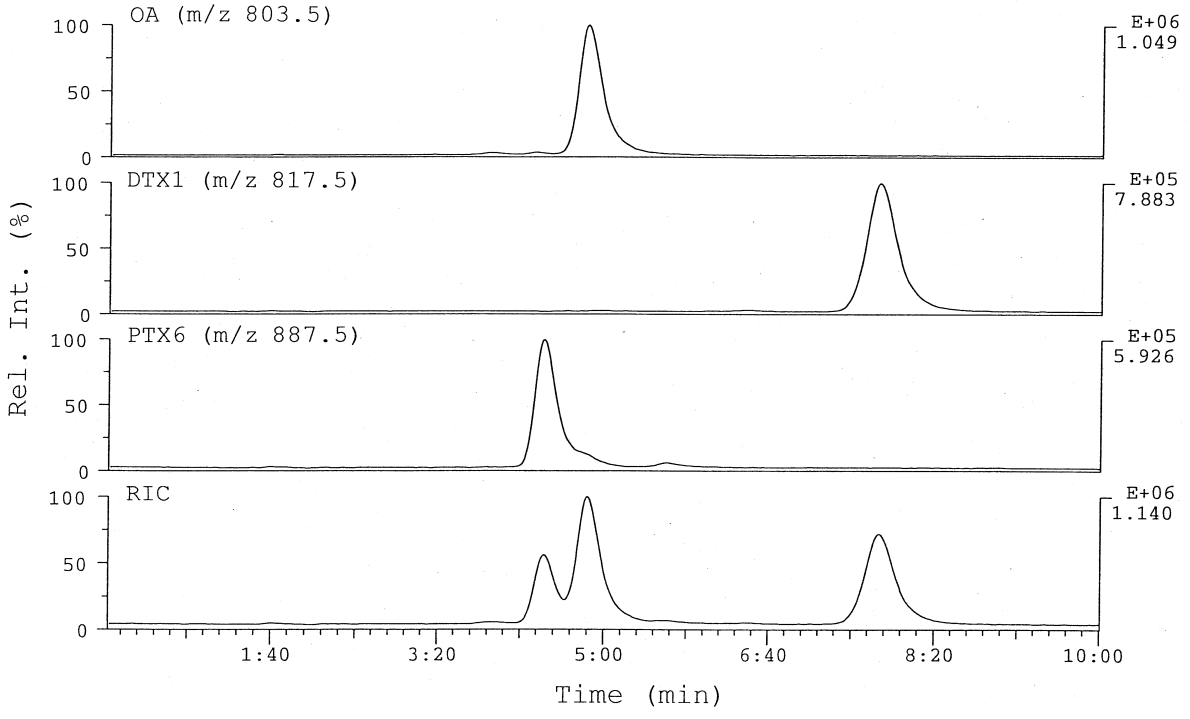


Fig. 3. LC–MS chromatograms for a mixture of OA, DTX1 and PTX6 standards obtained by negative SIM for $[M\text{-}H]^-$ ions of toxins. Column: Mightysil RP-18 (150 mm×2.0 mm I.D.); mobile phase: MeCN–water (7:3) with 0.1% acetic acid; flow-rate: 200 μl/min; column temperature: 35°C. RIC; reconstructed total ion chromatogram. A mixture of 2 ng of each toxin was injected.

0.1% acetic acid. Although acetonitrile–water (7:3, v/v) without additive gave sufficient ionization efficiency for toxins as well as that containing 0.1% acetic acid, acetonitrile–water (7:3, v/v) containing 0.1% acetic acid was selected as the mobile phase of LC–MS. During the optimization of MS parameters, it was observed that the ionization efficiency of toxins increased with increasing heated capillary temperature, however the intensity of mass spectrum of PTX6 decreased above 200°C. Therefore, the droplet desolvation of toxins in the heated capillary was carried out at 200°C.

Ionization of toxins by positive ESI mode was also surveyed with the same solvent used for negative ESI mode. Toxins yielded mass spectra exhibiting $[M+NH_4]^+$, $[M+Na]^+$ and $[M+H]^+$ as reported in the previous ion-spray detection [13,17,23] and ESI detection [11], however ionization efficiency was far lower than by negative ESI mode.

Fig. 3 shows a chromatogram of toxins obtained by negative-ion SIM detection at m/z 803, 817 and 887 after LC separation on a Mightysil RP-18 column. The separation factor (α) of OA/PTX6 and DTX1/OA was 1.23 and 2.07, respectively. Linearity of the peak area with increasing amounts of toxin was confirmed over a range from 10 pg to 30 ng (OA, $r^2=0.9996$; DTX1, $r^2=0.9996$; PTX6, $r^2=0.9998$) as shown in Fig. 4, indicating that it is possible to determine the toxins from 10 ng to 30 μg toxin per gram of midgut glands. The detection limit with the basis of a signal-to-noise ratio of 3:1 was 5 pg per injection. The detection limit in the present study is obviously better than that obtained by the SIM analysis on the ion-spray detection of OA (400 pg per injection) [14] and by the selected-reaction-monitoring (SRM) analysis on the ion-spray MS-MS detection of OA homologues (15 pg per injection) [22].

3.2. Application to bivalve samples

Table 1 shows the results of a recovery test for

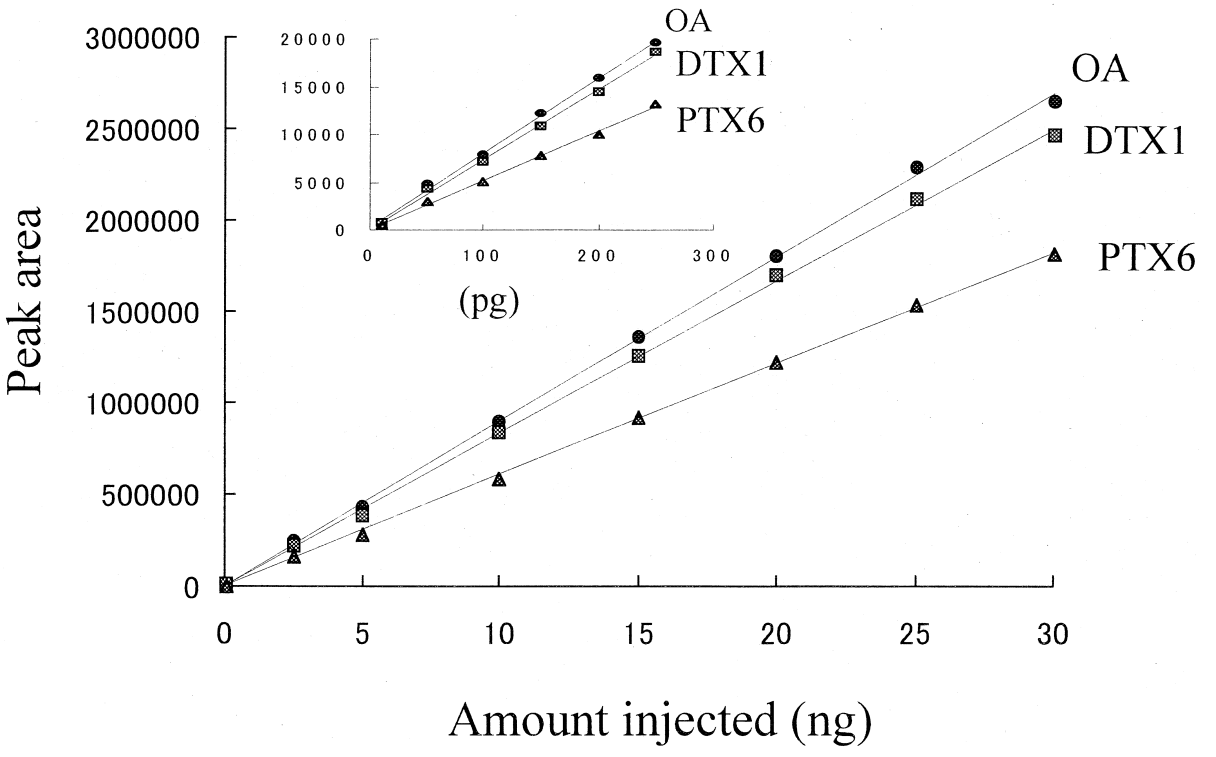


Fig. 4. Calibration curve for peak area of toxins obtained by SIM after LC separation. Each point is the mean of duplicate analyses.

Table 1
Recovery of toxins from spiked methanol–water (8:2, v/v) extract of midgut glands of bivalves

Toxins	Recovery (%) (mean±SD $n=6$)[a]
OA	102±2
DTX1	101±3
PTX6	86±2

[a] Mean±standard deviation. Recovery was calculated from the following samples: scallops with 0.2 μg of toxins per gram of midgut glands, mussels with 0.2 μg of toxins per gram of midgut glands, scallops with 1.0 μg of toxins per gram of midgut glands, mussels with 1.0 μg of toxins per gram of midgut glands, scallops with 4.0 μg of toxins per gram of midgut glands, mussels with 4.0 μg of toxins per gram of midgut glands.

toxins from the methanol–water (8:2, v/v) extracts of midgut glands spiked with standard toxins determined by SIM after LC separation. Extract prepared from non-toxic scallops and mussels showed no background peaks in the RIC. OA and DTX1 were almost completely recovered from the methanol–water (8:2, v/v) extracts of bivalve midgut glands. The recovery of PTX6 was slightly lower than that of OA and DTX1. The peak intensity of PTX6 co-injected with bivalve extracts was slightly lower than when only pure PTX6 was injected. This suggests that interfering compounds in bivalve extracts reduced ionization efficiency of PTX6.

Fig. 5 shows RIC of SIM at m/z 803, 817 and 887 obtained from the midgut glands of scallops collected at Mutsu Bay, Japan. Clear peaks of DTX1 and PTX6 were detected. Although a peak with m/z 803 was detected, the retention time of this peak (7.35 min) did not agree with that of standard OA (4.92 min) and DTX2 (5.47 min). This unknown compound (OAX) with m/z 803 was detected in the 1% NH_4OH–methanol (1:1, v/v) fraction which elutes acidic toxins (OA, DTX1, PTX6) when the midgut gland extracts were separated on an alumina B column. Although there is a possibility that OAX is an adduct ion (e.g. $[2M-H]^-$ of a molecular weight 402 compound) or a fragmentation ion from some

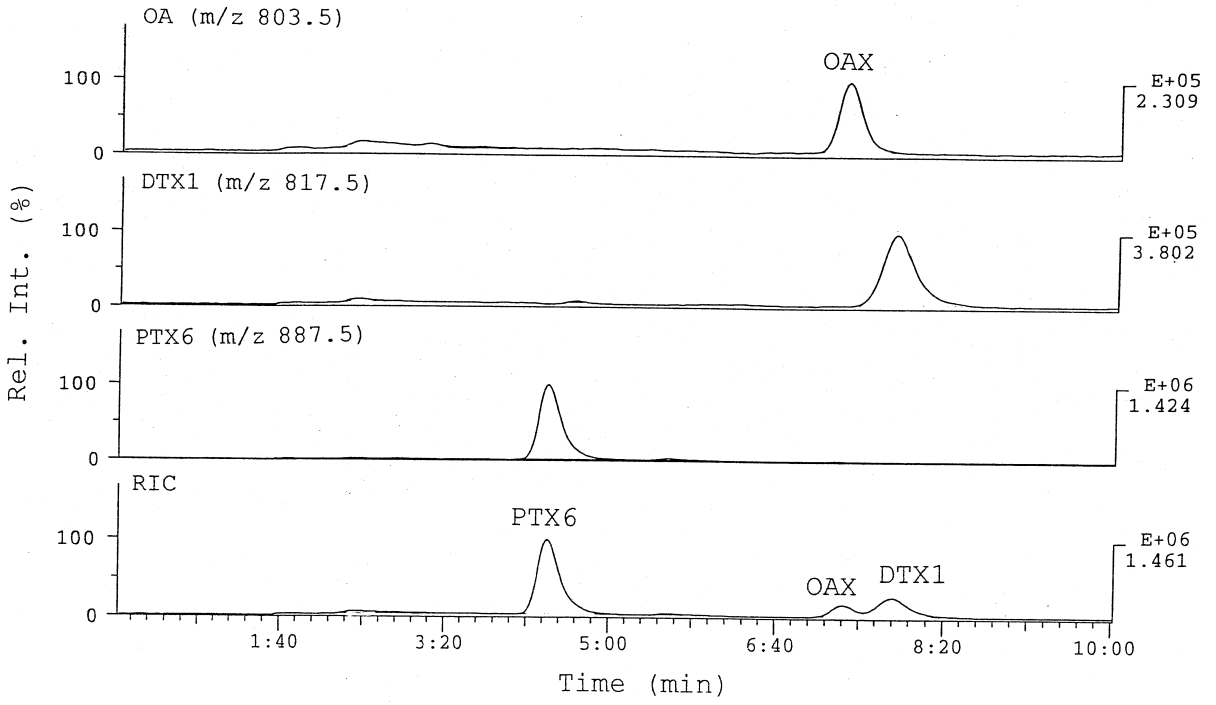


Fig. 5. LC–MS chromatograms of toxic scallop extracts collected at Mutsu Bay, Japan, on 29 May 1995 obtained by negative SIM for $[M-H]^-$ ions of toxins. Column: Mightysil RP-18 (150×2.0 mm I.D.); mobile phase: MeCN–water (7:3) with 0.1% acetic acid; flow-rate: 200 μl/min; column temperature: 35°C. RIC; reconstructed total-ion chromatogram.

Table 2
DSP toxin contents in midgut glands of bivalves collected at Mutsu Bay, Japan, in 1995 determined by LC–MS

Date	Sample	Toxin contents (μg/g)		
		OAX[a]	DTX1 (esterified DTX1[b])	PTX6
22 May	Scallop	0.29	0.41	2.58
29 May	Scallop	0.45	0.96	3.90
6 June	Scallop	0.08	1.36	5.71
19 June	Scallop	0.04	0.66 (0.46)	4.05
19 June	Mussel	0.06	4.13 (0.26)	ND

[a] OAX was determined by comparison with peak area of standard OA.
[b] Esterified DTX1 (DTX3) obtained from hydrolysis of hexane extracts. OA was not detected.

compound, the results in the present study suggest that the scallops were contaminated by an unknown OA homologue.

Table 2 lists the toxin contents determined by ESI–LC–MS in scallops and mussels collected at the same site in Mutsu Bay. The OAX contents were not in parallel with those of DTX1 and PTX6, implying that the source of OAX could be different from *D. fortii*. It is noteworthy that the toxin proportion is significantly different between scallops and mussels. The relative ratio of the toxin contents of esterified DTX1 (DTX3) to DTX1 in scallops was 0.70. The value was higher than that calculated in mussels (0.06). Although PTX6 was detected from scallops, it was not detected from mussels. Esterified DTX1 (DTX3) and PTX6 are converted from dinoflagellate produced DTX1 and PTX2, respectively, in bivalves [8–11,24]. The results obtained in the present study suggest that transformation kinetics from DTX1 to esterified DTX1 (DTX3) or from PTX2 to PTX6 in scallops is faster than in mussels.

In conclusion, ESI–LC–MS with heated capillary desolvation can successfully be applied to the determination of OA, DTX1 and PTX6. The ESI–LC–MS analysis of toxins clarified that the toxin profile was significantly different between scallops and mussels even when they were collected at the same site.

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References

[1] T. Yasumoto, Y. Oshima, M. Yamaguchi, Bull. Japan. Soc. Sci. Fish. 44 (1978) 1249–1255.
[2] T. Yasumoto, M. Murata, Chem. Rev. 93 (1993) 1897–1909.
[3] K. Terao, E. Ito, T. Yanagi, T. Yasumoto, Toxicon 24 (1986) 1141–1151.
[4] Y. Hamano, Y. Kinoshita, T. Yasumoto, J. Food Hyg. Soc. Japan 27 (1986) 375–379.
[5] H. Fujiki, M. Suganuma, H. Suguri, S. Yoshizawa, K. Takagi, N. Uda et al., Jpn. J. Cancer Res. 79 (1988) 1089–1093.
[6] M. Ishige, N. Satoh, T. Yasumoto, Bull. Hokkaido Inst. Public Health 38 (1988) 15–19.
[7] M. Daiguji, M. Satake, K.J. James, A. Bishop, L. MacKenzie, H. Naoki et al., Chem. Lett. (1998) 653–654.
[8] T. Yasumoto, M. Murata, J.S. Lee, K. Torigoe, in: S. Natori, K. Hashimoto, Y. Ueno (Eds.), Mycotoxins and Phycotoxins '88, Elsevier, Amsterdam, 1989, pp. 375–382.
[9] J.S. Lee, M. Murata, T. Yasumoto, in: S. Natori, K. Hashimoto, Y. Ueno (Eds.), Mycotoxins and Phycotoxins '88, Elsevier, Amsterdam, 1989, pp. 327–334.
[10] J.S. Lee, T. Igarashi, S. Fraga, E. Dahl, P. Hovgaard, T. Yasumoto, J. Appl. Phycol. 1 (1989) 147–152.
[11] T. Suzuki, T. Mitsuya, H. Matsubara, M. Yamasaki, J. Chromatogr. A 815 (1998) 155–160.
[12] H. Ogino, M. Kumagai, T. Yasumoto, Natural Toxins 5 (1997) 255–259.
[13] S. Pleasance, M.A. Quilliam, A.S.W. de Freitas, J.C. Marr, A.D. Cembella, Rapid Commun. Mass Spectrom. 4 (1990) 206–213.
[14] S. Pleasance, M.A. Quilliam, J.C. Marr, Rapid Commun. Mass Spectrom. 6 (1992) 121–127.
[15] J.C. Marr, T. Hu, S. Pleasance, M.A. Quilliam, J.L.C. Wright, Toxicon 30 (1992) 1621–1630.
[16] M.A. Quilliam, J. AOAC Int. 78 (1995) 555–570.
[17] R. Draisci, L. Lucentini, L. Giannetti, P. Boria, A. Stacchini, Toxicon 33 (1995) 1591–1603.
[18] R. Draisci, L. Lucentini, L. Giannetti, P. Boria, R. Poletti, Toxicon 34 (1996) 923–935.
[19] A. Gago-Martinez, J.A. Rodriguez-Vazquez, P. Thibault, M.A. Quilliam, Natural Toxins 4 (1996) 72–79.
[20] K.J. James, A.G. Bishop, M. Gillman, S.S. Kelly, C. Roden, R. Draisci et al., J. Chromatogr. A 777 (1997) 213–221.
[21] K.J. James, E.P. Carmody, M. Gillman, S.S. Kelly, R. Draisci, L. Lucentini et al., Toxicon 35 (1997) 973–978.
[22] R. Draisci, L. Lucentini, L. Giannetti, P. Boria, K.J. James, A. Furey et al., J. AOAC Int. 81 (1998) 441–447.
[23] R. Draisci, L. Giannetti, L. Lucentini, C. Marchiafava, K.J. James, A.G. Bishop et al., J. Chromatogr. A 798 (1998) 137–145.

[24] T. Suzuki, H. Ota, M. Yamasaki, Toxicon 37 (1999) 187–198.
[25] W.M. Niessen, J. Chromatogr. A 794 (1998) 407–435.
[26] H. Goto, T. Igarashi, R. Sekiguchi, K. Tanno, M. Satake, Y. Oshima et al., in: B. Reguera, J. Blanco, M.L. Fernández, T. Wyatt (Eds.), Harmful Algae, UNESCO, Santiago de Compostela, 1998, pp. 216–219.
[27] T. Hu, J. Doyle, D. Jackson, J. Marr, E. Nixon, S. Pleasance et al., J. Chem. Soc. Chem. Commun (1992) 39–41.
[28] J.S. Lee, T. Yanagi, R. Kenma, T. Yasumoto, Agric. Biol. Chem. 51 (1987) 877–881.
[29] M.L. Fernández, A. Míguez, E. Cacho, A. Martínez, Toxicon 34 (1996) 381–387.
[30] J. Zhao, G. Lembeye, G. Cenci, B. Wall, T. Yasumoto, in: T.J. Smayda, Y. Shimizu (Eds.), Toxic Phytoplankton Blooms in the Sea, Elsevier, Amsterdam, 1993, pp. 587–592.