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METABOLIC TRANSFORMATION OF DINOPHYSISTOXIN-3 INTO DINOPHYSISTOXIN-1 CAUSES HUMAN INTOXICATION BY CONSUMPTION OF *O*-ACYL-DERIVATIVES DINOPHYSISTOXINS CONTAMINATED SHELLFISH

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ABSTRACT — This paper describes for the first time a massive intoxication episode due to consumption of shellfish contaminated with 7-*O*-acyl-derivative dinophysistoxin-1, named Dinophysistoxin-3 (DTX-3). 7-*O*-acyl-derivative dinophysistoxin-1, a compound recently described in the literature, was found in shellfish samples collected in the Chilean Patagonia fjords. This compound does not inhibit Protein Phosphatases and also does not elicit the symptoms described for Diarrheic Shellfish Poisoning (DSP). The data showed here, give evidence of metabolic transformation of 7-*O*-acyl-derivative dinophysistoxin-1 (DTX-3) into Dinophysistoxin-1 (DTX-1, Methyl-Okadaic acid) in intoxicated patients. This metabolic transformation is responsible for the diarrheic symptoms and the intoxication syndrome showed by patients that consumed contaminated shellfish, which showed only the presence of 7-*O*-acyl-derivative dinophysistoxin-1. Patients fecal bacterial analysis for the presence of enteropathogens was negative and the mouse bioassay for DSP, performed as described for regulatory testing, was also negative.

The HPLC-FLD and HPLC-MS analysis showed only the presence of DTX-3 as the only compound associated to DSP toxins in the contaminated shellfish samples. No other DSP toxins were found in the shellfish sample extracts. However, the patient fecal samples showed DTX-1 as the only DSP toxins detected in fecal. Moreover, the patient fecal samples did not show DTX-3.

Since 7-*O*-acyl-derivative dinophysistoxin-1 (DTX-3) was the only compound associated to DSP toxins detected in the shellfish samples, an explanation for the diarrheic symptoms in the intoxicated patients would be the metabolic transformation of DTX-3 into DTX-1. This transformation should occur in the stomach of the poisoned patients after consuming 7-*O*-acyl-derivatives dinophysistoxin-1 (DTX-3) contaminated bivalves.

KEY WORDS: Dinophysistoxins *O*-acyl-derivatives, Dinophysistoxin 3, Dinophysistoxin 1, DSP human intoxication, DSP toxins

INTRODUCTION

Diarrheic Shellfish Poisoning (DSP), corresponds to the syndrome caused by diarrheic shellfish toxins (DSP toxins), characterized by generating a symptomatology that develop within three hours after the consumption of contaminated bivalves, entailing symptoms as diarrhea, nausea, vomits, and abdominal pain. If mild, the syndrome favorably evolves toward total recovery in a period ranging from 1 to 3 days, and no fatalities have been recorded (Yasumoto *et al*., 1978; Hamano *et al*., 1986; Aune *et al*., 1998; García *et al*.,

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2003).

The toxins involved correspond to a 38 carbons lypophilic polyether family, among which stand out Okadaic Acid (OA), Dinophysistoxin-1 (DTX-1) and Dinophysistoxin-2 (DTX-2), produced by dinoflagellates of the genre *Dinophysis sp* (Yasumoto *et al.*, 1979; Lee *et al.*, 1989; Yasumoto and Murata, 1993).

DSP toxins are accumulated through filtering in bivalves such as blue mussels, ribbed mussels and clams, which agglomerate 80% of the toxins in their digestive glands. Depending on the bivalves refill and natural depuration, the DSP toxins metabolism is modified inside the mollusk (Hu *et al.*, 1992a; Hu *et al.*, 1992b; Getal-Otero, 2000).

DTX-1 is esterified in the hydroxyl group of the 7 Carbon, by fatty acids ranging from tetradecanoic acid (C14:0) to docosahexaenoic acid (C22:6w3), being palmitic acid the most common fatty acid found in DTX-1, transformed to 7-*O*-acyl-derivatives of dinophysistoxin-1 (Dinophysistoxin-3, DTX-3) (Yasumoto *et al.*, 1985). DTX-1 and DTX-3 have been isolated only from shellfish samples, DTX-3 is absent in wild and cultivated phytoplankton samples (Suzuki *et al.*, 1999).

Poisoning produced every year by DSP toxins are relevant worldwide (Marr *et al.*, 1992; Fernández *et al.*, 1996; Vale and Sampayo 1999; Codier *et al.*, 2000). Nevertheless, in view of their similarity with other symptomatologies produced by enteropatogens, this type of poisoning is frequently associated with the one produced by *Vibrio parahaemolitic* and *Bacillus cereus*, both are found routinely in bivalve mollusks. Therefore, there is no accurate information linked to the annual DSP human poisoning episodes (Getal-Otero, 2000).

The underlying molecular mechanism of action associated to DSP toxins, is associated to their potent inhibitory action against ser/thre Protein Phosphatase 2A (PP2A), Protein Phosphatase 1 (PP1) and Protein Phosphatase 2B (PP2B), which requires high concentrations of DSP toxins to be inhibited (Bialojan and Takai, 1988; Rivas *et al.*, 2000). On the other hand, DTX-3 does not inhibit the Protein Phosphatase enzymes (Takai *et al.*, 1992) but this DTX-1 ester is straightforwardly hydrolyzed to DTX-1 by digestive enzymes such as lipases and esterases. The inhibitory effect of DSP toxins on PP2A has been studied in detail (Takai *et al.*, 1992; Sasaki *et al.*, 1994; Rivas *et al.*, 2000).

In Chile, outbreaks associated with DSP intoxications have occurred throughout Patagonian fiords since 1970 (Muñoz *et al.*, 1992; Uribe *et al.*, 2001; Garcia *et al.*, 2003). At present, the three most austral Regions (X, XI and XII) portray the endemic presence of DSP toxins (Lagos, 1998; Rivas *et al.*, 2000; Uribe *et al.*, 2001; Garcia *et al.*, 2003). Recently, DTX-3 has been identified in the X Region (García *et al.*, 2004) and DTX-1 in the XI-XII Regions (Uribe *et al.*, 2001, García *et al.*, 2003).

Okadaic acid (OA) and DTX-1 (Methyl Okadaic acid) have been shown to be potent tumor promoters, and considering that stomach, small intestine, and colon contain binding sites for OA, they could be implicated in the worldwide increase of gastrointestinal tumors (Suganuma *et al.*, 1988; Fujiki *et al.*, 1988). García *et al.*, 2003 and 2004, reported DTX-3 as the major compound associated to DSP toxins in Chilean shellfish samples, the content of DTX-3 normally exceed the international safe limit of 200 nanograms per gram of digestive glands in all shellfish samples analyzed.

In this paper we report the presence of DTX-3 as the only DSP toxins associated compound detected in contaminated shellfish. No other DSP toxins were found. Only the 7-*O*-acyl-derivative of dinophysistoxin-1 were detected in shellfish that was consumed by patients, whose ending into hospital with DSP intoxication symptoms. However, in the patients fecal samples only DTX-1 was detected. The data reported in this study show metabolic transformation of DTX-3 into DTX-1, this one occurs in the gut of poisoned patients that had consumed 7-*O*-acyl-derivatives dinophysistoxins contaminated bivalves.

MATERIALS AND METHODS

Reagents

Okadaic acid (OA) and Dinophysistoxin-1 (DTX-1), standard toxins, were obtained from SIGMA (Sigma Chemical Co, St, Louis, Mo, USA); 9-antryl-diazomethane (ADAM) was purchased from Molecular Probes (USA); deoxycholic acid (DOCA) was purchased from SIGMA (Sigma Chemical Co, St, Louis, Mo, USA); HPLC grade solvents (acetonitrile, acetone, methanol, dichloromethane, chloroform) were purchased from Merck (MERCK, Darmstadt, Germany); the SEP-PAK® Cartridges for solid phase extraction of Silica and C-18 were purchased from the Waters Corporation (Division of MILLIPORE, Milford, Ma. USA); dichloromethane and hexane, used for extraction and clean-up (Mallinckrodt, USA); glass distillation was used when solvent quality did not meet

the requirement of purity specified by standard operation procedures (SOP); water of high purity grade was obtained by elution through an ion exchange cartridge, and then by boiling for 2 hr with nitrogen bubbling.

Mouse Bioassay of DSP

The bioassay was done according toYasumoto et al. (1984).Twenty grams sample of homogeneized hepatopancreas is extracted thrice with 100 ml of acetone. The extracts are filtered, then the filtrated is collected and the solvent removed by rotary evaporation. The residue is made up to 20 ml with water and the suspension is extracted thrice with 50 ml of diethyl-ether. The combined organic layers are backwashed twice with small quantities of water and evaporated to dryness. As in the original procedure described above, the residue is resuspended in 1% Tween 60 solution to a concentration of 5 g hepatopancreas/ml Tween 60 prior to intraperitoneally injection into each of three mice (CF-1) weighting 18 g. Initially, 1 mouse unit (MU) was defined as the minimum dose of toxin required to kill a mouse within 48 hours.

Mussel extract for DSP toxins determination

The mussel extracts were obtained from shellfish samples collected on January 2003 from the XI Region of Chile. Two grams of the digestive glands were removed from *Mytilus chilensis* (Blue mussels), and homogenized and extracted two times with 3 ml of chilled 80% methanol, under mechanical stirring using a tissue tearor (BioHomogenizer M 133/2280, Biospec Products, Inc., Bartlesville, OK, USA). Then, the methanolic phase was centrifuged at 1,500 × g for 5 min, 2.5 ml of the supernatant were diluted with water to a final 26.66% methanol. 5 ml from this dilution were then transferred to a 250 mg C-18 SEP PAK® cartridge. The system was washed with 5 ml of 50% methanol (discard, to remove lipid components). Then 5 ml of pure methanol were added, in order to elute the DSP toxins, and this eluted fraction was evaporated to dryness under reduced pressure in a Speed Vac Plus (Savant, SC 210A, Farmingdale, NY. USA). The clean and dry extracts were used for derivatization with ADAM.

Alkaline Hydrolysis of esterified DSP toxins

The hydrolysis of the extracts was done according to García *et al.* (2004). In this case, 2.5 ml of 0.5 N NaOH in 90% methanol solution was added to a 2.5 ml aliquot of the 80% methanol extract of each shellfish sample. The mixture was heated for 50 min a 75°C. After evaporating the methanol from the reaction mixture, the aqueous layers was acidified with 0.5 N HCl and then extracted two times with 5 ml of diethyl ether. After evaporating the solvent, the extracts were dissolved in 2.5 ml of 80% methanol and extracted twice with 2.5 ml of hexane. Additionally, 1 ml of 0.2% acetic acid was added to the methanolic solution, the resulting toxins solution was extracted with 4.0 ml of dichloromethane. This eluted fraction was evaporated to dryness under reduced pressure in a SpeedVac Plus (Savant, SC 210 A, Farmingdale, NY) and then derivatized with ADAM.

Sample preparation

The collected feces samples were mixed with 2 ml of 80% dichloromethane, immediately frozen and stored at −20°C for later analysis. After thawing, the samples were extracted in a similar way as shellfish samples described above. Finally, samples were filtered through 5,000 M.W. cut-off microcentrifuge filters (Ultrafree-MC C3GC, Millipore Corp., MA, USA). The clean and dry extracts were used for derivatization with ADAM.

Derivatization of DSP phycotoxins with ADAM

The derivatization of standards and extracts of toxin samples, were carried out according to García *et al.* (2003). Briefly, the solid mussel extract residues or standards were treated with a freshly prepared solution of 0.1% ADAM (in 100 µl of acetone and 400 µl of methanol) (Lee *et al.*, 1987). After 1 hr at 25°C in the dark, the samples were evaporated to dryness and the residues were diluted in 200 µl CH_2Cl_2/hexane, 1:1 (v/v) and then transferred into a 500 mg Silica gel SEP PAK® cartridge. Each system was washed successively with 5 ml of CH_2Cl_2/hexane, 1:1 (v/v) and 5 ml CH_2Cl_2. Finally, eluted with 5 ml of CH_2Cl_2/methanol, 1:1 (v/v). The last fractions were evaporated to dryness, each one dissolved in 1 ml methanol and then 10 µl samples were injected and analyzed by HPLC with fluorescence on line detection (HPLC-FLD).

Chemical Analysis by HPLC-FLD method

The HPLC chemical analysis were performed on a Shimadzu Liquid Chromatograph System equipped with a pump (Shimadzu LC-6A), Rheodyne injector (7725i Rheodyne. Cotati, Ca. USA) and a Fluorescence detector (Shimadzu RF-535). 10 µl of samples toxins derivatives were injected on a reverse phase column Supelcosil LC-18 (5 µm; 25 cm × 4 mm) (Supelco, Bellefonte, PA. USA). An isocratically

mobile phase of $CH_3CN/CH_3OH/H_2O$ 8:1:1 (v/v) with a flow rate of 1 ml/min were run at room temperature. The excitation and emission wavelengths were set at 365 and 415 nm respectively. Peaks in the resulting chromatograms were identified by comparison with the retention times of DSP phycotoxin analytical standards. This method corresponds to a High Performance Liquid Chromatography with fluorescence on line detection (HPLC-FLD) with pre-column derivatization.

HPLC-MASS analysis

HPLC-mass spectrometry was performed using a Hewlett Packard Model 1050, Series liquid chromatograph coupled to a VG PLATAFORM mass spectrophotometer (FISON Instrument) equipped with an atmospheric pressure chemical ionization (APcI) device. The liquid chromatography flow was introduced into the interface without any splitting. High purity 99% nitrogen was used as the nebulizing gas (ca 0.2 l/min). Separation of samples was achieved on a C-18 column (Supelcosil 5 µm, ODS 4,6 × 150 mm. SUPELCO) at room temperature at isocratic conditions. The mobile phase was 90% aqueous acetonitrile at a flow rate of 0.2 ml/min. A potential of 2.5 kV was applied at the corona and voltage cones of 30 and 50 V were used. The source temperature was set at 350°C and the probe temperature was set at 150°C. The negative ion mode was used with a full scan between 300 to 1200 *m/z*. Mass calibration of the instrument was carefully performed and checked.

RESULTS

On January 2004, in San Jose de la Mariquina (39 ° 30 '/39 ° 45 ' Lat. 72 ° 45 '/73 ° 00 ' Long), 35 miles from Valdivia, the main City around, 26 patients: six children (13.3 ± 4.32 years)(mean ± SEM, N=6) and twenty adults (32.4 ± 8.65 years) (mean ± SEM, N=20) arrived to the local hospital with symptoms of digestive intoxication.

They reported to had ate Chilean blue mussels (*Mytilus chilensis*) bought in a beach community nearby (Mehuín 39 ° 15 '/39 ° 30 ' Lat.; 73 ° 00 '/73 ° 15 ' Long.) just an hour before. Patients showed the following symptoms: nauseas (46.1%), vomiting (30.7%), abdominal pain (76.9%) and diarrhea (57.7%). None of the patients showed significant hemodynamic changes. The most frequent complains were associated with intensive abdominal pain and fluid diarrhea. While a rapid infusion of intravenous saline treatment was performed, patient's fecal samples were taken.

The patient's fecal bacterial analysis was negative to the presence of enteropatogens, and the samples were sent for DSP toxins analysis. Samples were analyzed by DSP mouse bioassay (Yasumoto *et al.*, 1984), which was also negative. The mouse bioassay is one of the official methods to detect DSP toxins in shellfish extract, according to national and international regulations.

In parallel, every sample was derivatized with ADAM and analyzed by HPLC-FLD as described above (García *et al.*, 2003, 2004). Fig. 1 shows the chromatograms of the HPLC analysis of blue mussel extracts and fecal human samples derivatized with ADAM. In the shellfish extract sample, no signal of DSP toxins was detected (Fig. 1A).

In order to look for possible *O*-acyl-derivatives of dinophysistoxins, already described in high amounts in Chilean shellfish samples by García *et al.*, 2003 and 2004, half of these samples were previously treated with 0.5 N NaOH to promote alkaline hydrolysis of the possible *O*-acyl-derivatives of dinophysistoxins. After the alkaline hydrolysis, the derivatization reaction with ADAM was performed; this procedure yielded a single peak showing a retention time of 11:33 min (Fig. 1B), which is the same retention time of DTX-1 analytical standard (Fig. 1C). In feces samples extracts, with no alkaline hydrolysis and just after derivatization with ADAM, a peak corresponding to DTX-1 was immediately identified showing the same retention time of DTX-1 analytical standard (Fig. 1D). The average amount of DTX-1 found in patient fecal samples was in 4.24 ± 0.82 ng/g (mean ± SEM, N=26) of dry fecal sample. After alkaline hydrolysis treatment, feces samples did not show significant increase of DTX-1 concentration, showing that in feces patient extract, dinophysistoxins-3 (DTX-3) the *O*-acyl-derivative dinophysistoxin-1 was totally absent (Table1).

In order to confirm these results, a new sampling and monitoring expedition was set out. The same DSP contaminated blue mussel samples were collected again and this time from their original extraction areas (described by the local fishermen whose sold the shellfish). The localities monitored were: Niniualac (45°02`90 " Lat, 74°21`94 " Long; Zone 1), Anna Pink (45°47`20 " Lat; 74°42`70 " Long.; Zone 2) and Pulluche (45°47 ' 87 " Lat; 74°26`72 " Long; Zone 3), all places located in XI Region near Puerto Aysén fjord (Fig. 2). Each of the identified samples showed amounts of DTX-3 above 200 ng/g of digestive glands.

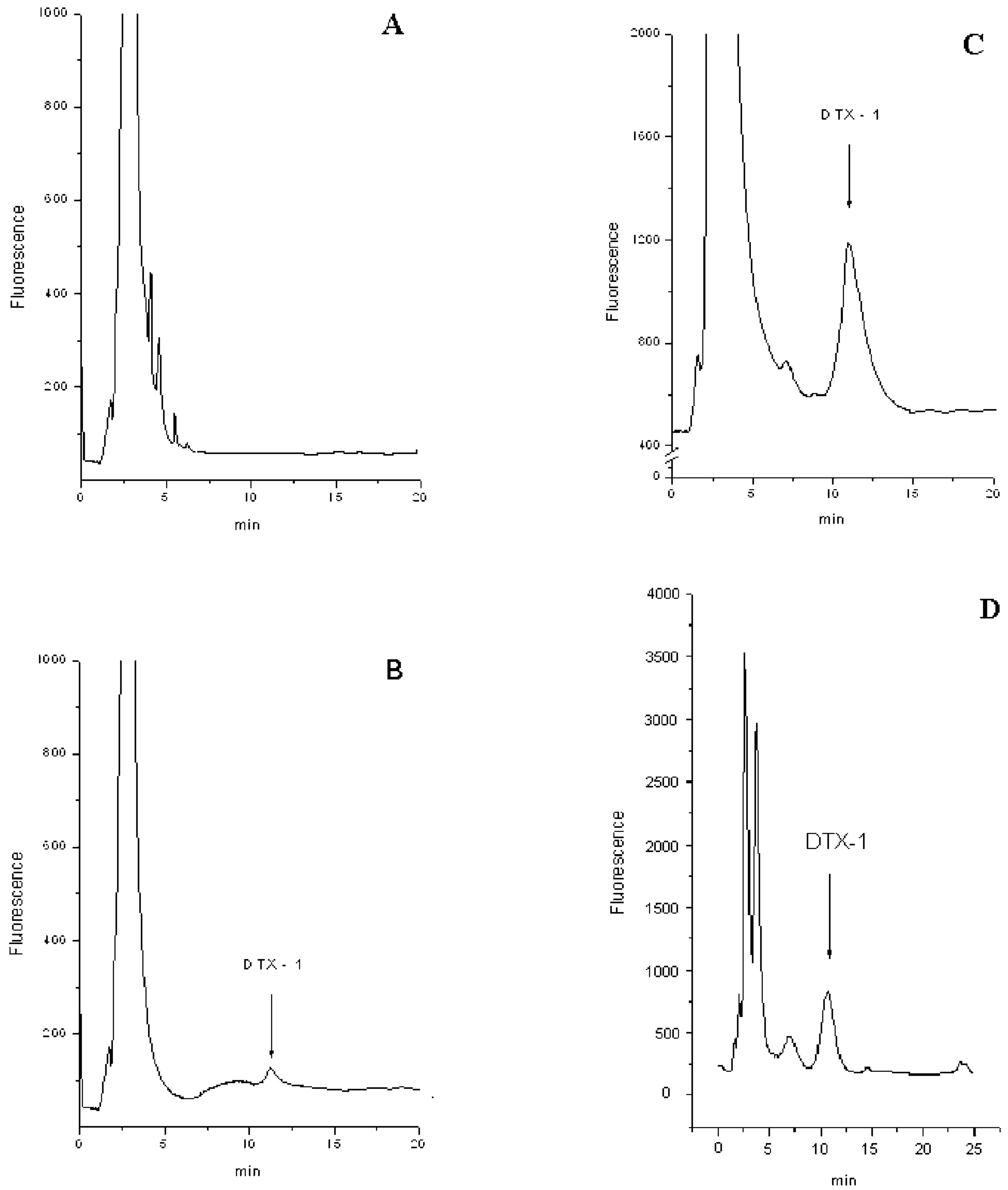

Fig. 1. Chromatograms of ADAM-DSP phycotoxins. A.- Blue mussel extract without alkaline hydrolysis. B.- Blue mussel extract after 0.5 N NaOH treatment (alkaline hydrolysis). C.- Dinophysistoxin-1 analytical standard (DTX-1). D.- Patients fecal samples at five hours after intoxication.

The shellfish samples collected in Niniualac gave 216.8 ± 19.4 ng/g (mean ± SEM, N=8) of digestive glands, Anna Pink 260.1 ± 24.7 ng/g (mean ± SEM, N=8) of digestive glands and Pulleche zone 316.1 ± 17.5 ng/g (mean ± SEM, N=8) of digestive glands (Table 1).

The mass spectra showed in Fig. 3, correspond to de HPLC-MS analysis before and after the alkaline hydrolysis of the blue mussel sample extracts collected in the Aysén area. The mass spectra, obtained from full-scan spectra, were acquired in the negative ion peak centroid mode, over the mass ranges from 300 to 1200 *m/z* for dinophysistoxins. The figure 3A, shows the mass chromatogram before alkaline hydrolysis. It displays a major molecular ion peak at 1055 *m/z*, corresponding to DTX-3 (7-*O*-acyl-derivatives of dinophysistoxin-1). In this case, the 7-OH in DTX-1 was esterified with palmitic acid, showing the *O*-acyl-derivatives dinophysistoxin most frequently reported in shellfish samples (Yasumoto *et al*., 1985; García *et al*., 2003 and 2004).

Fig. 3B, shows the mass chromatogram after alkaline hydrolysis treatment. Here, a major molecular ion peak at 816 *m/z*, corresponding to DTX-1 is shown. Other molecular ion peaks were also observed (383 *m/z*, 397 *m/z*, 424 *m/z*, 635 *m/z*, 671 *m/z*) in this mass chromatogram, all of them corresponding to ion fragments of DTX-1. The most probable chemical structures of these fragments are displayed above the peak in the same figure. These data confirm the identity of DTX-1.

The only DSP toxin detected after the alkaline hydrolysis treatment was DTX-1, and this one is coming from the alkaline hydrolysis of its DTX-3 *O*-acyl-derivative extracted from the contaminated shellfish samples.

DISCUSSION

The contaminated shellfish samples analyzed showed DTX-3 amount in the range of 200 ng of DSP toxins/gram of digestive glands, being the Pulleche zone, the one with highest concentration 316.1 ± 17.5 ng/gr (mean ± SEM, N=8) of digestive glands (TABLE I).

The presence of DTX-3 in Chilean shellfish is not unusual, moreover, all samples measured by our laboratory in the last five years, have shown DTX-1 after alkaline hydrolysis treatment of the shellfish extract, this DTX-1 is coming from the 7-*O*-acyl-derivatives of dinophysistoxin, making DTX-1 the mayor DSP toxin in Chilean shellfish samples (Uribe *et al*., 2001; Lagos, 2002; García *et al*., 2004). The first report of DSP toxins in Chilean shellfish samples was published in 1993; the authors also described DTX-1 as the major DSP toxin in Chilean mussel samples (Zhao *et al*., 1993).

It is well known that, over long periods, contaminated mollusks can modify DSP toxins through enzymatic processes, allowing their esterification with fatty acids. This metabolic transformation generates products like the 7-*O*-acyl-derivatives of dinophysistoxins (Suzuki *et al*., 1999; Vale and Sampayo *et al*., 2002). Untill now the published data report that DSP sterified toxins do not inhibit Protein Phosphatase activity and therefore show low toxicity in mammalian, this due to esterification of the hydroxyl group in toxin C-7. The covalent binding generates a steric impediment to the carboxyl group, which is determinant for the inhibition of Protein Phosphatases (Takai *et al*., 1992).

The liquid chromatographic mass spectrometry

Table 1. DSP toxins analysis before and after alkaline hydrolysis treatment.

Samples	Not hydrolized		Hydrolized	
	OA (ng/g)	DTX-1 (ng/g)	OA (ng/g)	DTX-1 (ng/g)
Blue mussels				
Zone 1	n.d.	n.d.	n.d.	216.8 ± 19.4
Zone 2	n.d.	n.d.	n.d.	260.1 ± 24.7
Zone 3	n.d.	n.d.	n.d.	316.1 ± 17.5
Human Feces	n.d.	4.24 ± 0.82	n.d.	4.39 ± 0.70

n.d. = no detected
OA = okadaic acid
DTX-1 = dinophysistoxin-1
ng/g = nanograms/grams of digestive glands.

technique performed in this study, unambiguously shows DTX-3 in shellfish samples and DTX-1 in patient fecal samples, DTX-1 was also found in the shellfish extracts after alkaline hydrolysis treatment. The molecular ion of 1055 *m/z* detected before the alkaline hydrolysis, witch correspond to DTX-3, the 7-*O*-acyl-derivatives of dinophysistoxin-1, demonstrated that fatty acid is the acylating compound of the DTX-1 ester.

The results in this paper show for the first time a massive human intoxication produced by consumption of bivalves contaminated with DTX-3. This *O*-acyl-derivative dinophysistoxin-1 was not detected by the mouse bioassay official method, and consequently the shellfish was authorized for sale at the local markets.

Since only *O*-acyl-derivative dinophysistoxin-1

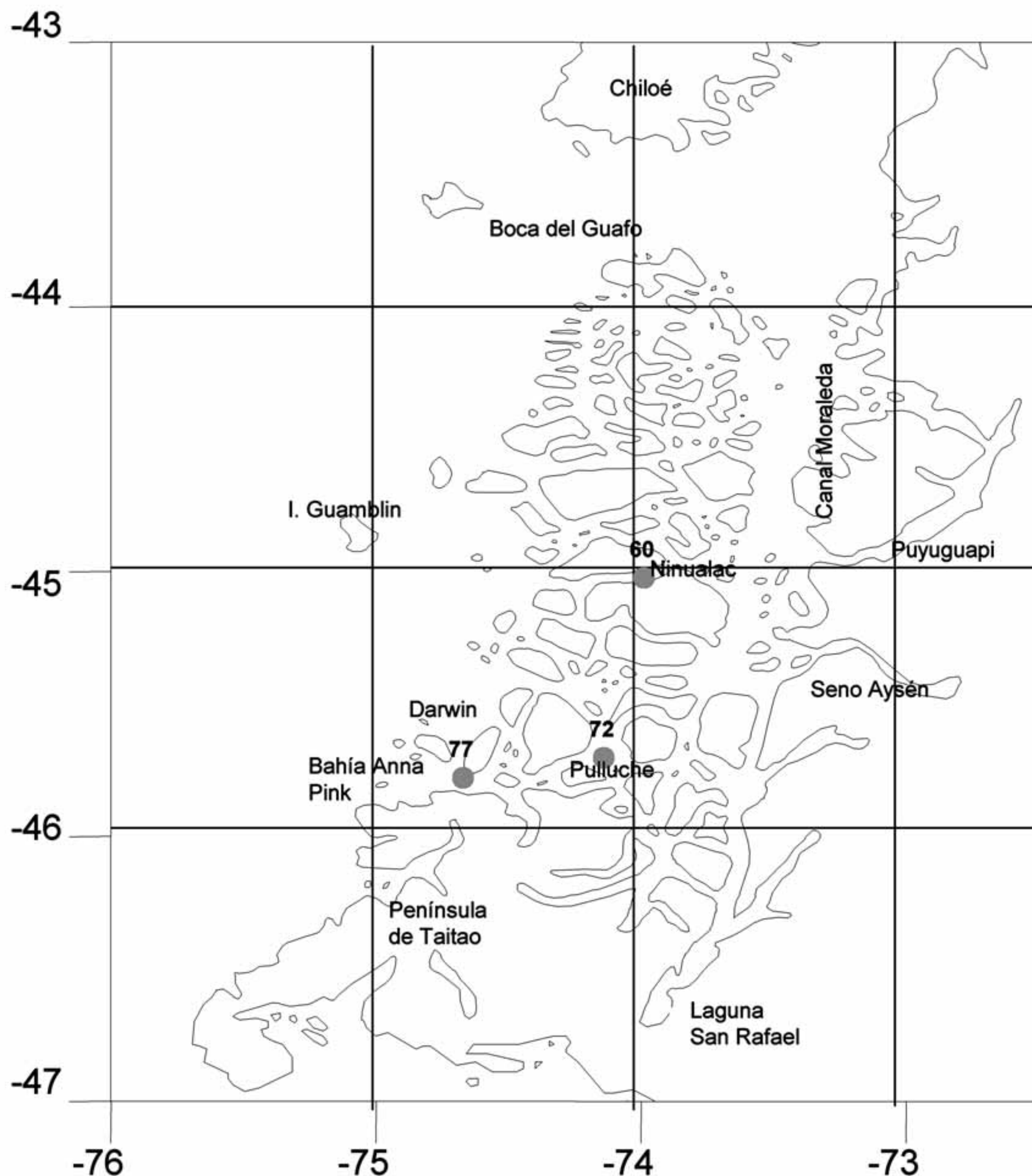

Fig. 2. Aysén Region map, Chile. ● show the littoral places were the DSP contaminated shellfish were collected by the monitoring program after intoxication.

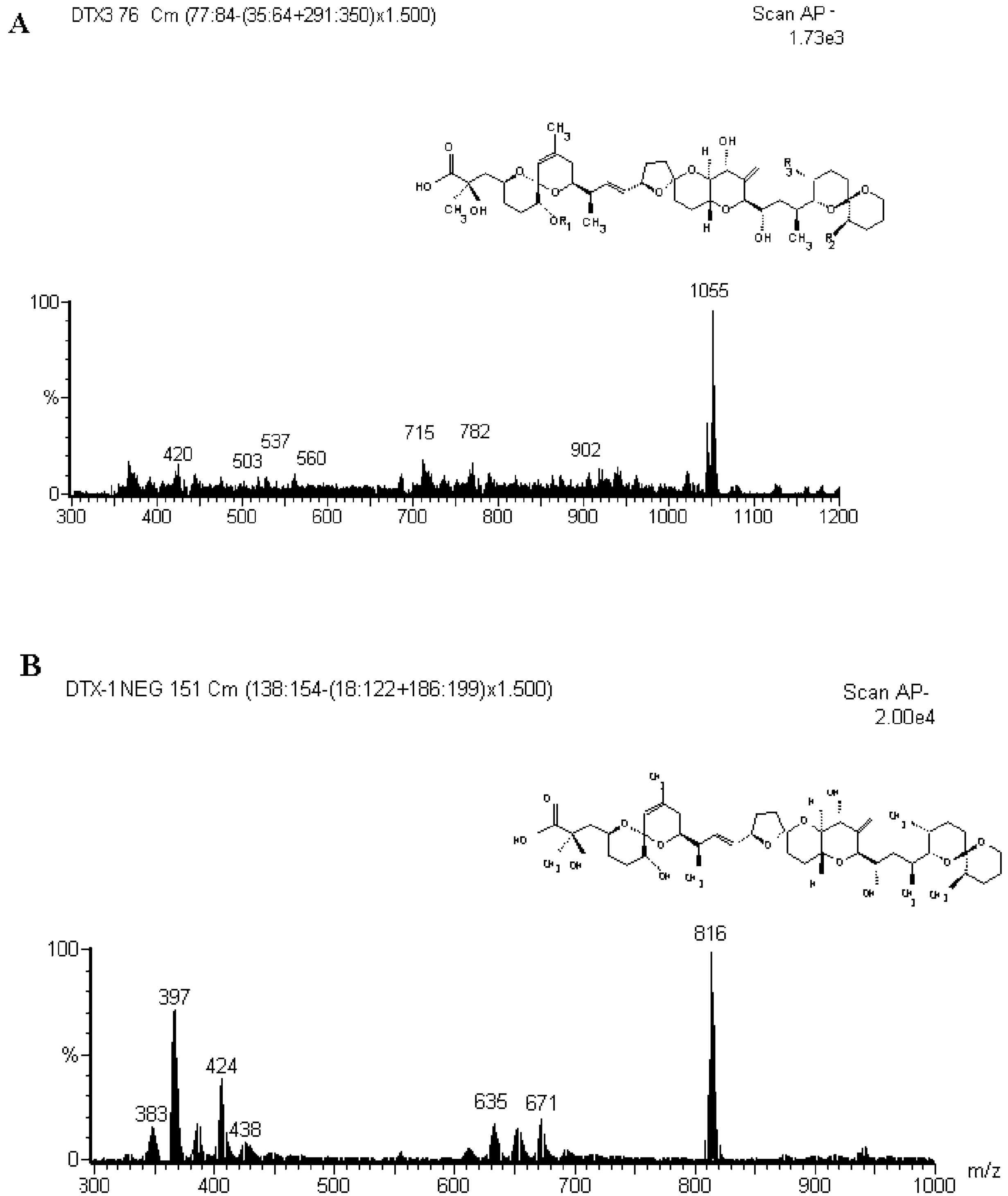

Fig. 3. HPLC-MS analysis of blue mussel samples. A.- Mass spectrum of a partial purified fraction of blue mussel sample before alkaline hydrolysis. The molecular ion peak of 1055 *m/z* correspond to DTX-3. B.- Mass spectrum of blue mussel extract after alkaline hydrolysis treatment. The molecular ion peak of 816 *m/z* correspond to DTX-1.

was detected in shellfish sample extracts, and patient fecal samples showed DTX-1 as the only DSP toxins detected and not a trace of DTX-3 was found; the possible explanation for the diarrheic symptoms and the intoxication syndrome showed by the intoxicated patients, is the metabolic transformation of *O*-acyl-derivative dinophysistoxin-1 (DTX-3) into Dinophysistoxin-1 (DTX-1, Methyl-Okadaic acid) by patients themselves.

Negative results in bacterial analysis, seeking for the presence of enteropatogens in patients fecal samples and negative mouse bioassay for DSP, strengthens this hypothesis. Furthermore, since HPLC–FLD and HPLC-MS analysis showed the presence *O*-acyl-derivative dinophysistoxin-1 (DTX-3) as the only compound associated to DSP toxins in shellfish samples, the metabolic transformation of DTX-3 into DTX-1 should occur in the stomach of the poisoned patients.

Enzymes such as lipases and esterases, that normally digest daily nutrients and xenobiotics, are capable to hydrolyze DTX-3, converting it into DTX-1 a DSP toxin, responsible for the intoxication syndrome associated to DSP described by the patients. The fast - less than 2 hr intoxication effect- reported by intoxicated patients, also strength the hypothesis of DSP intoxication, since developed faster than an intoxication caused by enteropatogens, which need an incubation period of at least 12 hr to produce diarrheic symptoms.

Literature points out that DTX-3 is only available in DSP contaminated shellfish as a product of the ester acylation of DTX-1. It is a provocative thought to consider this reaction as a defense mechanism developed by mollusks, since the inhibition activity effect of DTX-1 on mollusks Protein Phospatases is as potent as on human (Rivas *et al*., 2000).

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