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# First evidence of Dinophysistoxin-1 ester and carcinogenic polycyclic aromatic hydrocarbons in smoked bivalves collected in the Patagonia fjords

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**Abstract**

Diarrhetic shellfish poisoning (DSP) is a gastrointestinal disease caused by fat-soluble polyether toxins produced by dinoflagellates and accumulated in shellfish. Up to the present, only four fat-soluble polyethers have been known as diarrhetic shellfish toxins. Among them,*** Okadaic acid, Dinophysistoxin-1, Dinophysistoxin-2 and Dinophysistoxin-3. Outbreaks associated with DSP have occurred in the Chilean Patagonia fjords since 1970.

Native people, who live in small communities close to the southern fjords, smoke fresh shellfish. During this popular smoking procedure, they impregnate the shellfish with polycyclic aromatic hydrocarbons, as incomplete combustion products, which are potent carcinogenic compounds, this product is sold in local markets without phycotoxins analysis or inclusion in any monitoring program.

The present paper shows, DSP phycotoxins quantitation, using high performance liquid chromatography with fluorescent and mass spectrometric detection and the measurements of polycyclic aromatic hydrocarbons by gas chromatography with mass detection, in smoked shellfish samples. The presence of Dinophysistoxin-3, the Dinophysistoxin-1 ester (7-*O*-acyl-derivatives of dinophysistoxin-1), was assessed in all shellfish samples analyzed. The 7-OH in Dinophysistoxin-1 was esterified with palmitic fatty acid. The shellfish meat contains seven polycyclic aromatic hydrocarbons, among them fluoranthene, phenanthrene, anthracene, pyrene and benzo[a]pyrene. The gas chromatography-mass spectrometry analysis showed four of the six most frequent carcinogenic polycyclic aromatic hydrocarbons reported. The content of benzo[a]pyrene in the Razor Clam and Ribbed Mussel were 78.61 and 4.94 ng/g of shellfish dry weight, respectively. In both cases the benzo[a]pyrene amounts were greater than the acceptable tolerance limits of 1 μg/kg of sample. The Razor Clam samples also show amount further above the maximum label regulated by FAO/WHO (10 μg/kg). The presence of both type of compounds in the smoked shellfish samples analyzed, correspond to a dangerous combination, where the polycyclic aromatic hydrocarbons are carcinogenic compounds by themselves and DTX-1, is a potent tumor promoter.


*Keywords:* Diarrhetic shellfish poisoning toxins; Dinophysistoxin-1; Dinophysistoxin-2; Polycyclic aromatic hydrocarbons; Patagonia fjords; Chile

## 1. Introduction

Fujiki et al. (1987), showed that an application of okadaic acid (OA) on mouse skin induces irritation and

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induction of ornithine decarboxylase activity, suggesting that this compound could be a tumor promoter. Suganuma et al. (1988) demonstrated this potentially during a two-stage carcinogen experiment in rodents. OA appeared to be tumor promoter as potent as phorbol 12-tetradecanoate 13-acetate (TPA) for inducing papillomas and carcinomas when applied together with 7,12-dimethylbenzoanthracene (DMBA) to the skin of the back of 8-week-old female CD-1 mice (Suganuma et al., 1988). OA was first found as a cytotoxic agent when searching for antitumor drugs (Tachibana et al., 1981). Thus, the OA class phycotoxins are toxic to eukaryotic cells but not to the prokaryotic (Aonuma et al., 1991). Recently, OA and its derivatives Dinophysistoxin-1 (DTX-1) and Dinophysistoxin-2 (DTX-2), named like these because were found associated to the dinoflagellates of the genus *Dinophysis*, are considered diarrhetic (Terao et al., 1986; Hamano et al., 1986) and tumorogenic phycotoxins (Fujiki and Suganuma, 1993).

Dinophysistoxin-1 is the methyl derivative of OA and Dinophysistoxin-3, the DTX-1 ester (7-*O*-acyl-derivatives of dinophysistoxin-1). The 7-OH in DTX-1 can be esterified with fatty acids ranging from tetradecanoic acid (C14:0) to docosahexaenoic acid (C22:6w3), palmitic acid being the most common fatty acid found in DTX-3 (Yasumoto et al., 1985). DTX-1 and DTX-3 have been only isolated from shellfish samples, DTX-3 is absent in wild and cultivated plankton samples, for this reason it has been proposed that the acylation of the 7-OH in DTX-1 only occur in shellfish.

The underlying mechanism of action, associated with these toxin activities, is explained mainly by their potent inhibitory action against ser/thre Protein Phosphatase 2A (PP2A), 1 (PP1) and 2B (PP2B), the last one, inhibited only at high concentrations of phycotoxin (Bialojan and Takai, 1988; Rivas et al., 2000). DTX-3 does not inhibit the enzymes (Takai et al., 1992) but is easily hydrolyzed to DTX-1 by digestive enzymes such as lipase. The inhibitory effect of Diarrhetic Shellfish Poisoning (DSP) phycotoxins on Protein Phophatase 2A have been studied in details (Takai et al., 1992; Sasaki et al., 1994; Rivas et al., 2000).

DSP is a worldwide distributed gastrointestinal illness which shows a rapid-onset intoxication involving consumption of shellfish containing toxins produced by dinoflagellate of the genera *Dinophysis* spp. and *Prorocentrum* spp (Yasumoto et al., 1978; Yasumoto and Murata, 1993). The characteristic symptoms are gastrointestinal disorders such as diarrhea, nausea, vomiting and abdominal pain.

DSP is relevant from a health viewpoint not only because of its acute effects, but also because of its potential chronic effects which are not fully understood. Regarding acute effects, the gastroenteritis caused by DSP toxins has a favorable evolution toward total recovery in 2–3 days, and no fatalities have been described. Regarding chronic effects, OA and DTX-1 have been shown to be potent tumor promoters, and given that the stomach, small intestine, and colon have binding sites of OA, this could be implicated in the growth of gastrointestinal tumors (Suganuma et al., 1988; Fujiki et al., 1988). The mutagenetic (Aonuma et al., 1991) and immunotoxic effects due to a marked suppression of interleukin-1 (IL-1) production have also been described for these phycotoxins (Hokama et al., 1989). The role of tumor promoter of DSP phycotoxins have been demonstrated after a single application of the carcinogen DMBA, where the 80% of mice population resulted with tumor at the 30th week of treatment. By other hand, the tumor incidence in mice treated with either DMBA alone or OA alone was less than 10% (Suganuma et al., 1988).

Outbreaks associated with DSP have occurred in the Patagonia fjords since 1970 (Muñoz et al., 1992; Uribe et al., 2001; García et al., 2003). Actually, the three austral Regions of Chile show the endemic presence of DSP phycotoxins (Lagos, 1998; Rivas et al., 2000; Uribe et al., 2001; García et al., 2003).

To perform quantitative analysis of DSP phycotoxins in shellfish samples in Chile is very important: first, because these toxins are endemic present in molluscs of southern Regions, second the regulatory mouse bioassay, in the way it is done in Chile is only a qualitative assay, so the level of toxins is never known. Third, although human symptoms of DSP appear relative mild when compared with the Paralytic Shellfish Poisoning (PSP), also endemic in the Patagonia fjords, necessity of paying more attention to the chronic effects of continued uptake of low doses of DSP phycotoxins is imperative, since these DSP toxins have described as potent tumor-promoter (Suganuma et al., 1988). Moreover, the native people from this southern part of Chile, by tradition and culture eat and sell smoked shellfish prepared by themselves in a traditional way, without any control or monitoring program. This smoked shellfish should contain the most dangerous combination, the carcinogenic polycyclic aromatic hydrocarbons, the tumor initiator and the DSP phycotoxins, the tumor promoters.

The present paper shows the first evidence of Dinophysistoxin-1 ester (DTX-3) presence in smoked shellfish samples collected in Patagonia fjords. Moreover, the same shellfish extracts showed the presence of carcinogenic polycyclic aromatic hydrocarbons in tissues of shellfish, as chemical pollutants of the smoked procedure commonly used by local people. The presence of DSP phycotoxins and carcinogenic compounds both in the same shellfish sample, should be cause for concern for human consumption and should result in the implementation of monitoring programs of these smoked shellfish.

## 2. Materials and methods

### 2.1. Reagents

OA and Dinophysistoxin-1 (DTX-1) standard toxins were obtained from SIGMA (Sigma Chemical Co, St Louis, MO, USA), 9-antryldiazomethane (ADAM) was purchased

from Funakoshi Pharmacy (Tokyo, Japan). Deoxycholic acid (DOCA) was purchased from SIGMA (Sigma Chemical Co, St Louis, MO, USA). HPLC grade solvents (acetonitrile, acetone, methanol, chloroform) were purchased from Merck (MERCK, Darmstadt, Germany). The SEP-PAK® Cartridges for solid phase extraction of Silica and $C_{18}$, were purchased from Waters Corporation (Division of MILLIPORE, Milford, MA, USA). Dichloromethane and hexane used for extraction and clean-up were pesticide residue grade (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, suitable for PAHs analysis, was obtained by elution through an ion exchange cartridge and then by boiling for 2 h with nitrogen bubbling.

### 2.2. Mussel extract for DSP toxins determination

The smoked mussel extracts were obtained from samples purchased in a local market in April of 2001 from the X Region, Two grams of digestive glands were removed from *Mytilus chilensis* Hupe, (Blue mussel), *Aulacomya ater* (Ribbed mussel) and *Tangelus dombeii* (Razor clam), then were 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 was diluted with water to a final 26.66% methanol. From this dilution, 5 ml 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 to elute the DSP toxins, 5 ml of pure methanol was added, this eluted fraction was evaporated to dryness under reduce pressure in a Speed Vac Plus (Savant, SC 210A, Farmingdale, NY, USA). The clean and dry extracts were used for derivatization with ADAM.

### 2.3. Alkaline hydrolysis of esterified DSP toxins

The hydrolysis was done according to Suzuki et al. (1999). In this case, 2.5 ml of 0.5N 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 kept to 75 °C for 50 min. After evaporating the methanol from the reaction mixture, the aqueous layers was acidified with 0.5N HCl and then extracted two times with 5 ml diethyl ether. After evaporating the solvent, the extracts were dissolved in 2.5 ml 80% methanol and extracted twice with 2.5 ml hexane. Additional, 1 ml of 0.2% acetic acid was added to the methanolic solution, the resulting toxins solution was extracted with 4.0 ml of dichromethane. This eluted fraction was evaporated to dryness under reduced pressure in a Speed Vac Plus (Savant, SC 210 A, Farmingdale, NY) and then derivatized with ADAM.

### 2.4. Derivatization of DSP phycotoxins with ADAM

The derivatization of standards and sample toxins, were carried out according to Uribe et al., 2001. 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 h at 25 °C in the dark, the sample was evaporated to dryness and the residue was diluted in 200 μl $CH_2Cl_2$/hexane, 1:1 (v/v) and then transferred into a 500 mg Silica gel SEP PAK® cartridge. The 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 fraction was evaporated to dryness, dissolved in 1 ml methanol and then 10 μl was injected and analyzed by HPLC with fluorescent on line detection (HPLC-FLD).

### 2.5. 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), a rheodyne injector (7725i Rheodyne, Cotati, CA, USA) and a fluorescence detector (Shimadzu RF-535). Toxin derivatives of 10 μl were injected on a reversed 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 correspond to a High Performance Liquid Chromatography with fluorescent on line detection (HPLC-FLD) with pre-column derivatization.

### 2.6. Sample preparation for chemical analyses with gas chromatography and mass spectrometry

The smoked shellfish tissues were homogenized with 2 g of sodium sulfate anhydrous and 100 μl internal standard, perdeuterated PAHs, were added to the smoked shellfish and then extracted with 180 ml dichloromethane for 8 h under reflux with Soxhlet extraction apparatus. This extract was reduced to a 1 ml volume for Kuderna-Danish concentrators at 60 °C. Then 10 ml hexane was added and saponified with 50 ml of ethanolic solution of potassium hydroxide 1N, this mixture was under reflux for 2 h. The digest was liquid–liquid extracted with 20 ml $H_2SO_4$ and two times with 25 ml of *n*-hexane. The upper layer was filtrated and reduced to a 1 ml volume with Kuderna-Danish concentrators and clean up with silica gel/aluminum oxide/sodium sulfate anhydrous columns. The system was washed successively with 20 ml of *n*-hexane (fraction 1, discarded), with 20 ml *n*-hexane–dichloromethane 8:1 (v/v) (fraction 2, discarded) and 20 ml

*n*-hexane-dichloromethane 1:1 (v/v) (fraction 3). The last fraction was concentrated to 1 ml using Kuderna-Danish concentrators and kept in an amber glass vial. One microliter were analyzed by GC-MS (Maruya et al., 1997).

### 2.7. Gas chromatography-mass spectrometry analyses (GC-MS)

The aromatic fraction was analyzed by gas chromatography coupled to a mass selective detector (GC-MS). An HP 6890 PLUS gas chromatograph equipped with a programmable temperature vaporizing inlet (PTV) and coupled to an HP 5973 mass selective detector (Hewlett-Packard, Palo Alto, CA, USA) was used. The injector temperature was maintained at 250 °C in a pulsed splitless mode. A GC program temperature ramp from 60 °C for 4 min and then at a rate of 10 °C/min up to 300 °C was used to afford the best separation of HAPs by using a capillary HP-5 MS column, 30 m × 0.32 mm i.d. × 0.25 μm film thickness (Hewlett-Packard, Palo Alto, CA, USA). The MSD was operated under Single Ion Monitoring mode (SIM). The standard mixture used contain, acenaphthylene (Ac), fluorene (Flu), phenanthrene (P), anthracene (A), pyrene (Py), benzo[a]anthracene (BaA), chrysene (Chry), benzo[b]fluoranthene (Bbf), benzo[k]fluoranthene (Bkf), benzo[a]pyrene (BaPy), indene[1,2,3-cd]pyrene (IPy), dibenzo[a,h]anthracene (DahA) and benzo[ghi]perylene (BgPer) and they were quantified on a dry weight basis relative to perdeuterated PAHs added to the methylene chloride extracts. Unresolved peaks of benzo(k)- and (b)fluoranthenes are indicated as ΣBF. Total PAHs correspond to the sum of the parent compounds. Analytical protocols were validated by using certified sediment SRM 1941a (NIST) and certified tissue SRM 2974 (NIST). The recoveries of PAHs on the matrix were between 40 and 100%. The relative percent difference (RPD) for analytical duplicates were less than 25%. Detection limits for PAHs were 0.020 ng/g dry weight for both matrices analyzed (Baumard et al., 1999).

### 2.8. 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 nubulizing gas (ca 0.2 l/min). Separation of the sample was achieved on a C-18 column (Supelcosil 5 μm, ODS 4.6 × 150 mm SUPELCO) at room temperature and in 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 100 and 900 *m/z*. Mass calibration of the instrument was carefully checked.

## 3. Results

The smoked shellfish sampling was performed in one of our expedition to the Patagonia fjords, where we bought in the local market of Castro City, three different species of smoked shellfish that are currently commercially available. The species were, *Mythilus chilensis* (Blue mussel), *Aulacomya ater* (Ribbed mussel) and *Tangelus dombeii* (Razor clam). In order to test the presence of diarrhetic phycotoxins in smoked shellfish and at the same time to look for the presence of carcinogenic PAHs, both type of compounds were analyzed in the samples.

Fig. 1A shows the chromatographic run of smoked Blue mussel extract esterifies with ADAM, a fluorescent chromophorus, and detected by HPLC-FLD as described by García et al. (2003). Here, not a single signal of DSP toxins was observed. But, when the sample was treated with 0.5N NaOH, producing an alkaline hydrolysis before the derivatization with ADAM, a single peak with a retention time at 11:41 min appears showing the same retention time of DTX-1 standard (Fig. 1B).

From the structural point of view, DTX-1 is a polyether with a 38 backbone carbon skeleton and six pendant methyl groups. It is characterized by the presence of oxolane and oxane rings, which form spiro or trans-fused systems. This chromatogram corresponds to on line fluorescent detection of a pre-column derivatization procedure and it is the one obtained in the regular method developed for detection of DSP phycotoxins as routine analysis. Table 1 shows the amounts of DTX-1 measured (nanograms by grams of digestive glands) in the three shellfish samples analyzed before and after the alkaline hydrolysis.

The mass spectra showed in Fig. 2A correspond to de HPLC-MS analysis of the Ribbed mussel extract before the alkaline hydrolysis. 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 DTX phycotoxins. The mass chromatogram displays a major molecular ion peak at 1055 *m/z*, this one corresponds to DTX-3 (7-*O*-acyl-derivatives of dinophysistoxin-1). In this case, the 7-OH in DTX-1 is esterifies with palmitic acid, showing the DTX-3 phycotoxin most frequently reported in shellfish samples (Yasumoto et al., 1985).

Fig. 2B shows the mass spectra of a purified fraction of the Ribbed mussel sample after the treatment with NaOH methanolic solution, in order to obtain a controlled alkaline hydrolysis of DTX-3. The mass spectra were obtained also from full-scan spectra acquired in the negative ion mode over the mass range from 300 to 1000 *m/z*. Here, two prominent molecular ion peaks are observed, one of them corresponding to the molecular ion of DTX-1 (816 *m/z*) and a second one of

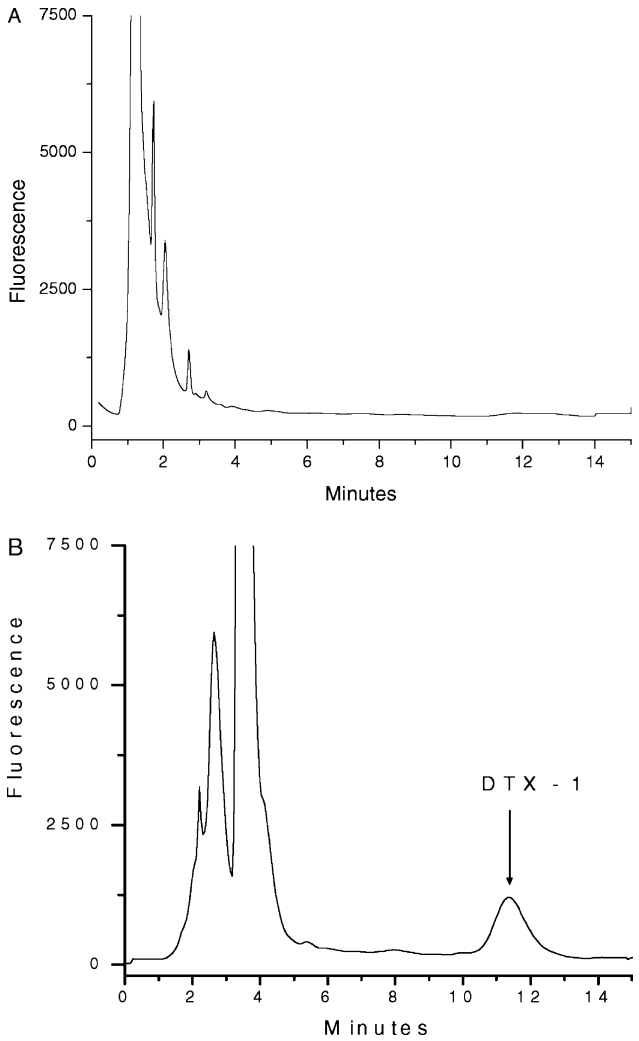


Fig. 1. Chromatograms of ADAM-DSP phycotoxin from Blue mussel extract. (A) Sample extract without alkaline hydrolysis. (B) Sample extract after 0.5N NaOH treatment.

529 *m*/*z* which correspond to a ion fragment of DTX-1, with the most probable chemical structure showed above the peak in the same mass spectra (Fig. 2B). This one also confirm the identity and purity of DTX-1, now coming from the alkaline hydrolysis of DTX-3 found in the sample before the NaOH methanolic solution treatment (Fig. 2A).

The samples tested for polycyclic aromatic hydrocarbons included the ribbed mussel, blue mussel and razor clam that showed DTX-1 content. Gas chromatography mass spectrometric determination was performed. The extracts showed the presence of: fluorene, phenanthrane, anthracene, pyrene, benzo[b]fluoranthrene, benzo[a]pyrene, dibenzo[a,h]anthracene and benzo[g,h,i]perylene (Table 2). Being fluorene and phenantrene plus anthracene the PAH compounds which showed the highest content in nanograms per grams of shellfish dry weight. Benzo[a]pyrene was found in the Razor

Table 1
DSP toxins analysis before and after alkaline hydrolysis

| Samples | Toxin digestive glands | | | |
|---|---|---|---|---|
| | Not hydrolized | | Hydrolized | |
| | OA (ng/g) | DTX-1 (ng/g) | OA (ng/g) | DTX-1 (ng/g) |
| Blue mussel | n.d. | n.d. | n.d. | 180.5 |
| Ribbed mussel | n.d. | n.d. | n.d. | 221.0 |
| Razor clams | n.d. | n.d. | n.d. | 120.0 |

n.d., No detected; OA, okadaic acid; DTX-1, dinophysistoxin-1; DTX-3, dinophysistoxin-3; ng/g, nanograms/grams of digestive glands.

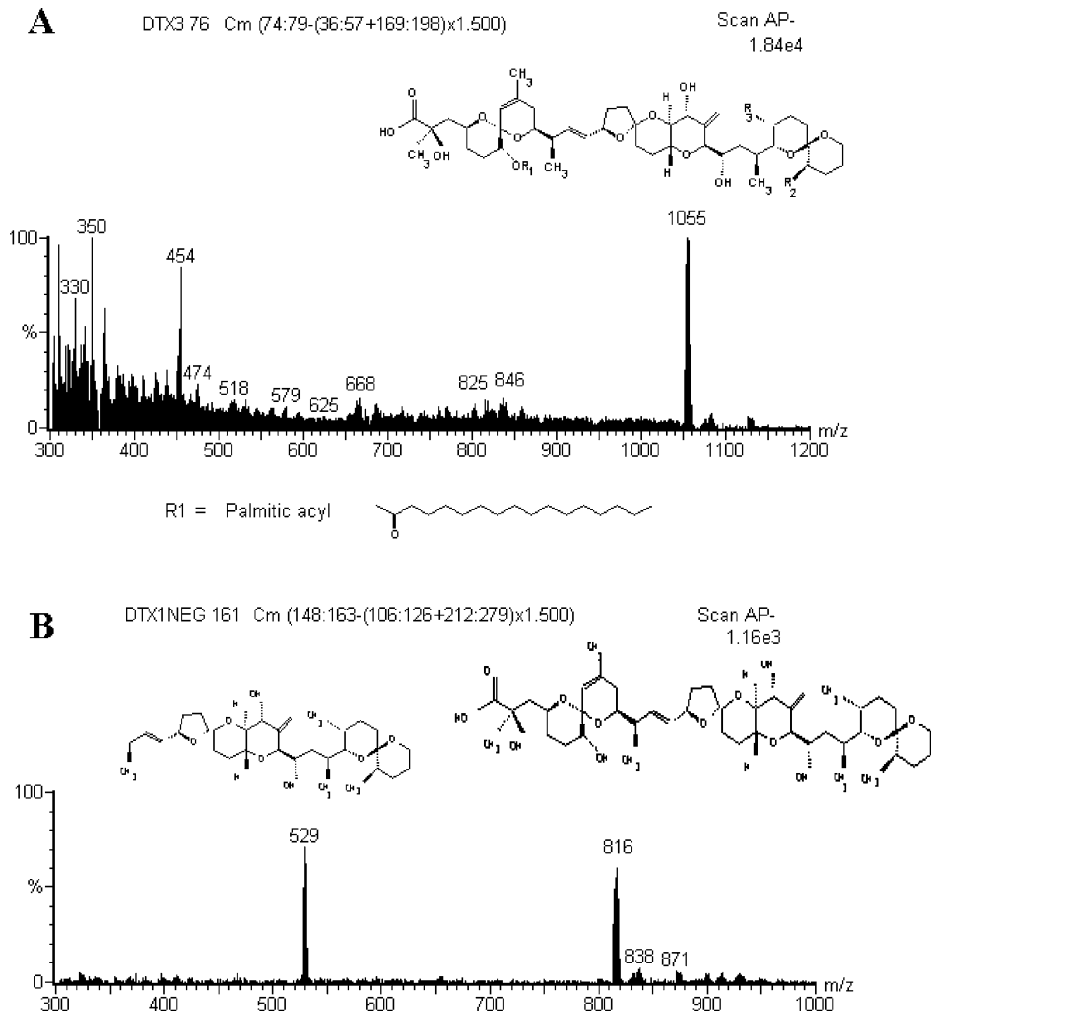


Fig. 2. HPLC-MS analysis of Ribbed mussel sample. (A) Mass spectrum of Ribbed mussel sample, the molecular ion peak of 1055 *m/z* correspond to DTX-3, obtained before the extract alkaline hydrolysis. (B) Mass spectrum of a purified fraction of the Ribbed mussel sample after alkaline hydrolysis, the molecular ion peak of 816 *m/z* correspond to DTX-1.

Table 2
PAHs total content found in Ribbed mussel, Razor clam and Blue mussel extracts

| Analites | Samples dry weight (ng/g) | | |
|---|---|---|---|
| | Blue mussel | Razor clams | Ribbed mussel |
| Acenaphthylene | n.d. | n.d. | n.d. |
| Fluorene | 2475.00 | 602.30 | 1395.99 |
| Phenanthrene + anthracene | 767.80 | 578.40 | 426.29 |
| Pyrene | 2.68 | 388.04 | 2.87 |
| Benzo[a]anthracene | n.d. | n.d. | n.d. |
| Chrysene | n.d. | n.d. | n.d. |
| Benzo[b]fluoranthene + benzo[k]fluoranthene | 47.08 | 29.97 | 8.91 |
| Benzo[a]pyrene | n.d. | 78.61 | 4.94 |
| Dibenzo[a,h]anthracene | 52.00 | 22.76 | 36.39 |
| Benzo[g,h,i]perylene | 55.39 | 43.83 | 51.70 |
| PAHs totals | 3399.95 | 1743.91 | 1927.09 |

n.d., No detected; ng/g, nanograms/grams; PAHs, polycyclic aromatic hydrocarbons.

clam and Ribbed mussel extracts, being in the Razor clam sample one of the highest values of the PAHs total contents in the shellfish extracts analyzed (Table 2). In both cases the benzo[a]pyrene amounts were grater than the acceptable tolerance limits (1 μg/kg of sample). In the Razor clam sample, the label was around eight times above the maximum label regulated by FAO/WHO (10 μg/kg). The PAHs content in the smoked shellfish varies considerably with species; Blue mussel contains the highest amount while the razor clams the lower. The PAHs total content found in Blue mussel is practically the double amount found in the other shellfish samples (Table 2).

The GC-MS chromatograms of the PAH standards and shellfish samples are shown in Fig. 3. Fig. 3A shows the retention times of the standards: Acenaphthylene (9:13 min), Fluorene (10:60 min), Phenathrene (12:62 min), Anthracene (12:73 min), Pyrene (15:63 min), Benzo[a]anthracene (18:21 min), Chrysene (18:32 min), Benzo[b]fluoranthene (20:39 min), Benzo[k]fluoranthrene (20:49 min), Benzo[a]pyrene (21:08 min), Dibenzo[a,h]anthracene (24:09 min), and Benzo[g,h,i]perylene (24:81 min). In the chromatogram, all of them are very well resolved, showing that the GC conditions used in this paper allowed to analyzed the majors PAHs normally found and described in different samples (Standard Reference Material, EPA 525). From the 13 polyaromatic hydrocarbons in the standard mixture used, only Indeno[1,2,3-cd]pyrene was not resolved in the run of 25 min described in this paper.

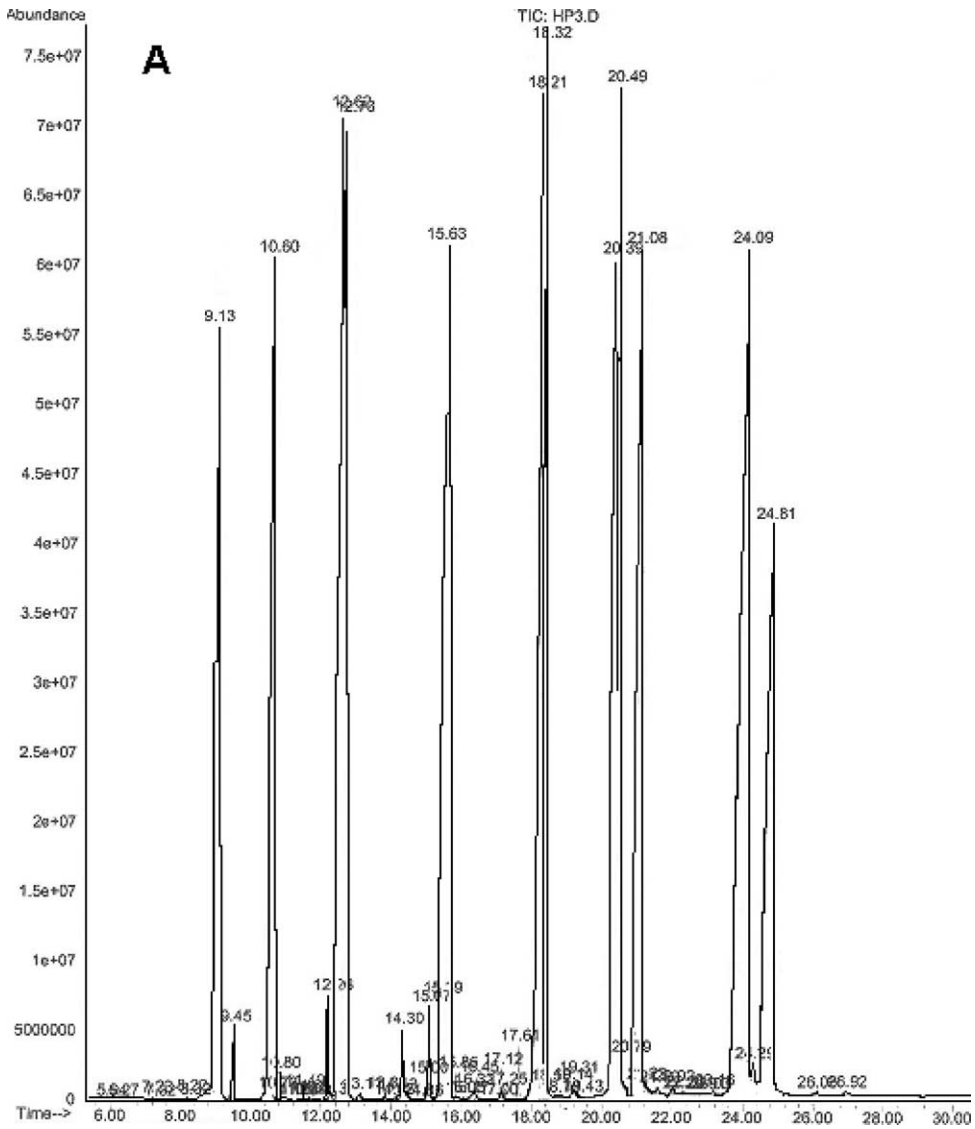


Fig. 3. Chromatograms of shellfish samples GC-MS analysis. (A) Standard mixture of 13 polycyclic aromatic hydrocarbons. (B) Ribbed mussel extract. (C) Razor clam extract. (D) Blue mussel extract.

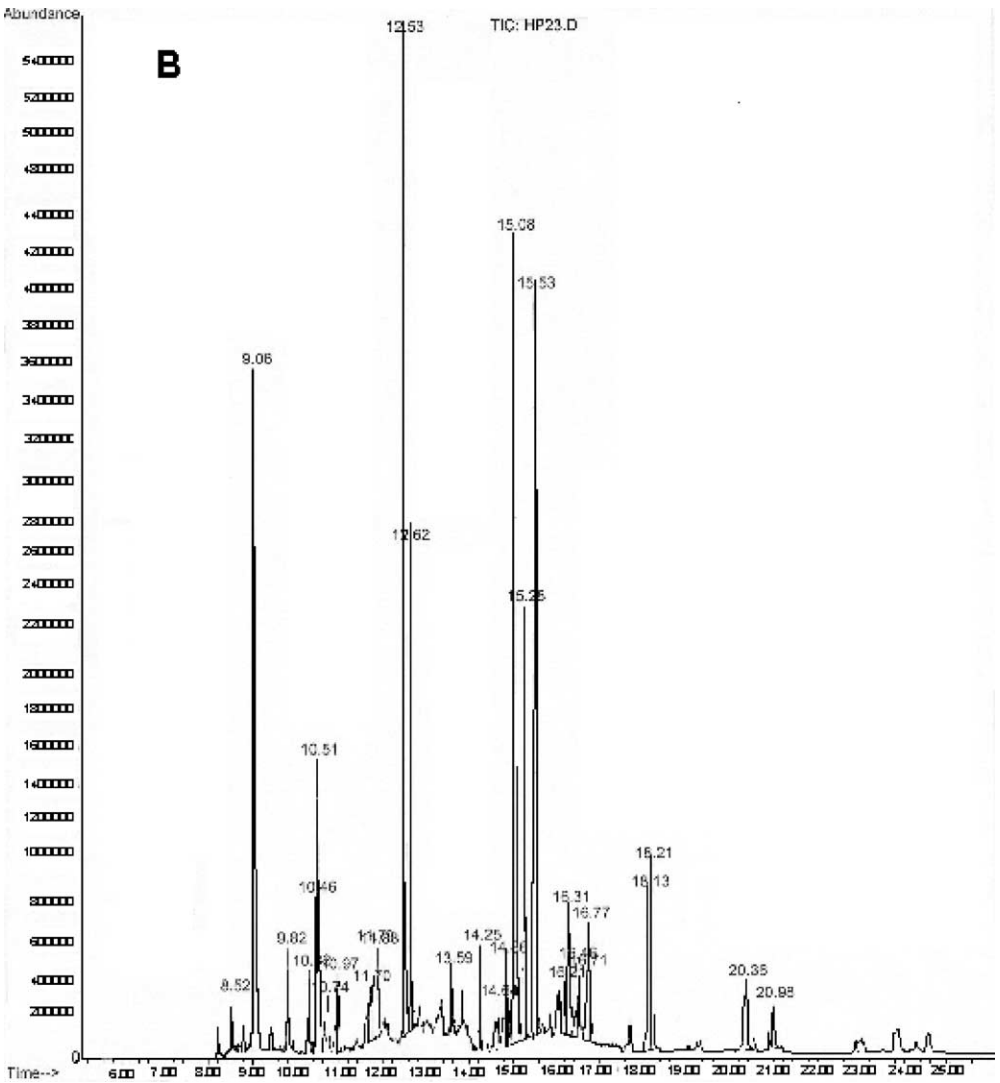


Fig. 3 (*continued*).

Fig. 3B–D shows the GC-MS chromatograms of the Ribbed mussel, Razor clam and Blue mussel samples, respectively. In all of them Fluorene appears as the major signal being the 72.4% (Ribbed mussel), 34.5% (Razor clam) and 72.8% (Blue mussel) of the PAHs total content of the sample. Also, these chromatograms show that Phenanthrene and Anthracene (the second major signal) and Benzo[b]fluoranthene and Benzo[k]fluoranthrene were resolved as one peak in the samples, for that reason both cases were informed together in Table 2.

In order to know the amount of PAHs extracted and also as internal standards, each extract was spiked with perdeuterated PAHs (Acenaphthene d-10; Phenanthrene d-10; Chrysene d-12 and Perylene d-12). All of them eluted very close to the perdeutered PAHs as was expected (Fig. 3B–D). The perdeuterated PAHs spiked extracts showed that the PAHs extraction method used in this paper allowed to extract in average 90% of the PAHs total content.

## 4. Discussion

The total amount of DTX-1 measured in the mussel samples analyzed (Table 1) were all around the international safe limits, 200 ng of DSP toxins/g of digestive gland, being the Ribbed mussel the highest value, 221 ng of DTX-1/g of digestive gland, this one above the safe limits. According to the DSP ban criteria in any monitoring program this shellfish it should be banned and its consumption prohibit. The presence of this DSP phycotoxin in Chilean shellfish is not unusual, since in practically all samples until now measured by our laboratory, DTX-1 is the major or the only

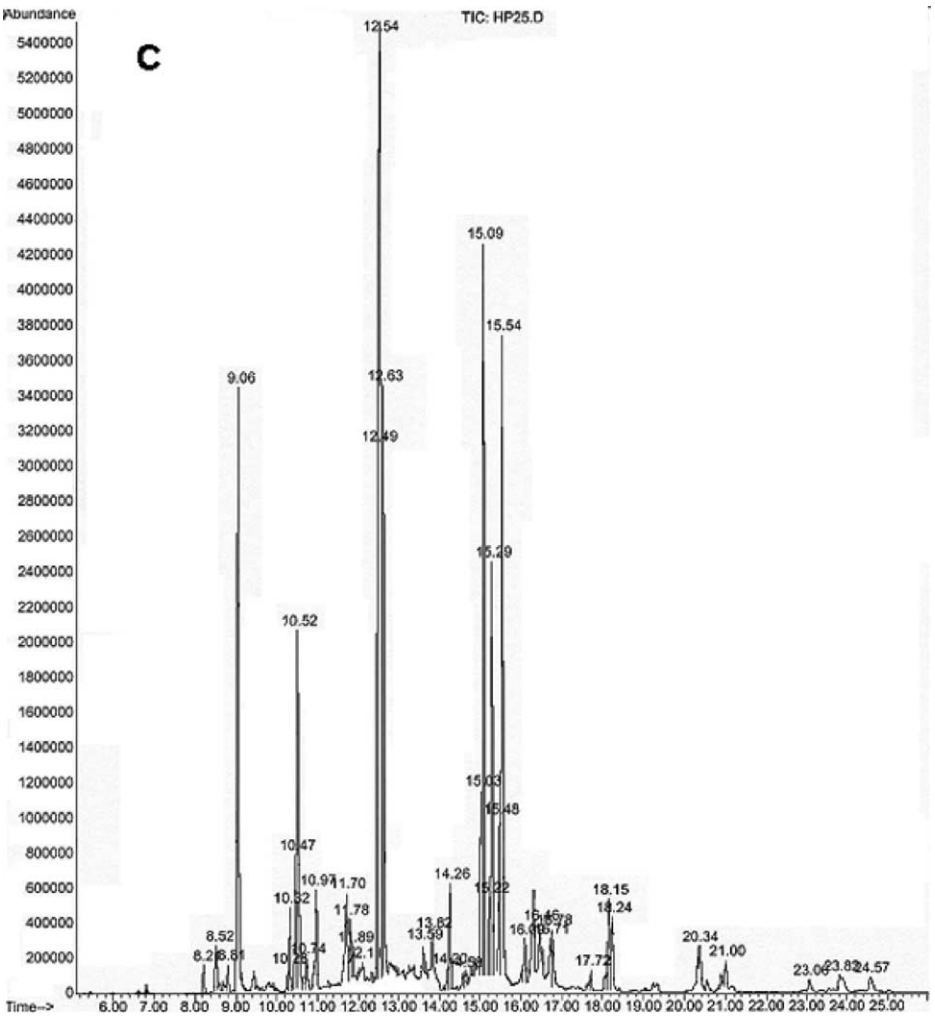


Fig. 3 (*continued*).

one DSP phycotoxins detected (Uribe et al., 2001; Lagos, 2002; García et al., 2003). This analysis correlate well with the one performed by Zhao et al. (1993), using a similar HPLC-FLD procedure. They also found DTX-1 as the major DSP phycotoxins in Chilean mussel samples (Zhao et al., 1993).

The liquid chromatographic mass spectrometry technique performed in this paper unambiguously identify the DSP phycotoxins DTX-3 and DTX-1. The later one was only seeing when the shellfish sample extract was alkaline hydrolyzed. 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 the fatty acid that is acylating the DTX-1 ester is palmitic acid, this acylation of DTX-1 is the most frequent found in shellfish samples worldwide. This is the first report of DTX-3 in Chilean shellfish samples.

The analysis of PAHs using gas chromatography-mass spectrometry analyses showed the presence of four from the six most frequently carcinogenic PAHs described in the literature (Gomaa et al., 1993). These were, Benzo[b]fluoranthene, Benzo[k]fluoranthrene, Benzo[a]-pyrene and Dibenzo[a,h]anthracene. Benzo[a]pyrene was present in the Razor clam (78.61 ng/g of dry weigh) and Ribbed mussel (4.94 ng/g of dry weigh) samples. In both cases the benzo[a]pyrene amounts were greater than the acceptable tolerance limits of 1 μg/kg of sample. The Razor clam sample also show amount further above the maximum label regulated by FAO/WHO (10 μg/kg). It should be noted, however, that none of the shellfish samples analyzed yielded concentration below the method quantitation limits and that the average and median concentrations measured were on the high side of acceptable tolerance limits. The results of our study show that levels of PAHs in smoked shellfish tissues are relative high. Moreover, the major carcinogenic components of smoked procedures (incomplete combustion products) were present in the samples. These elevated

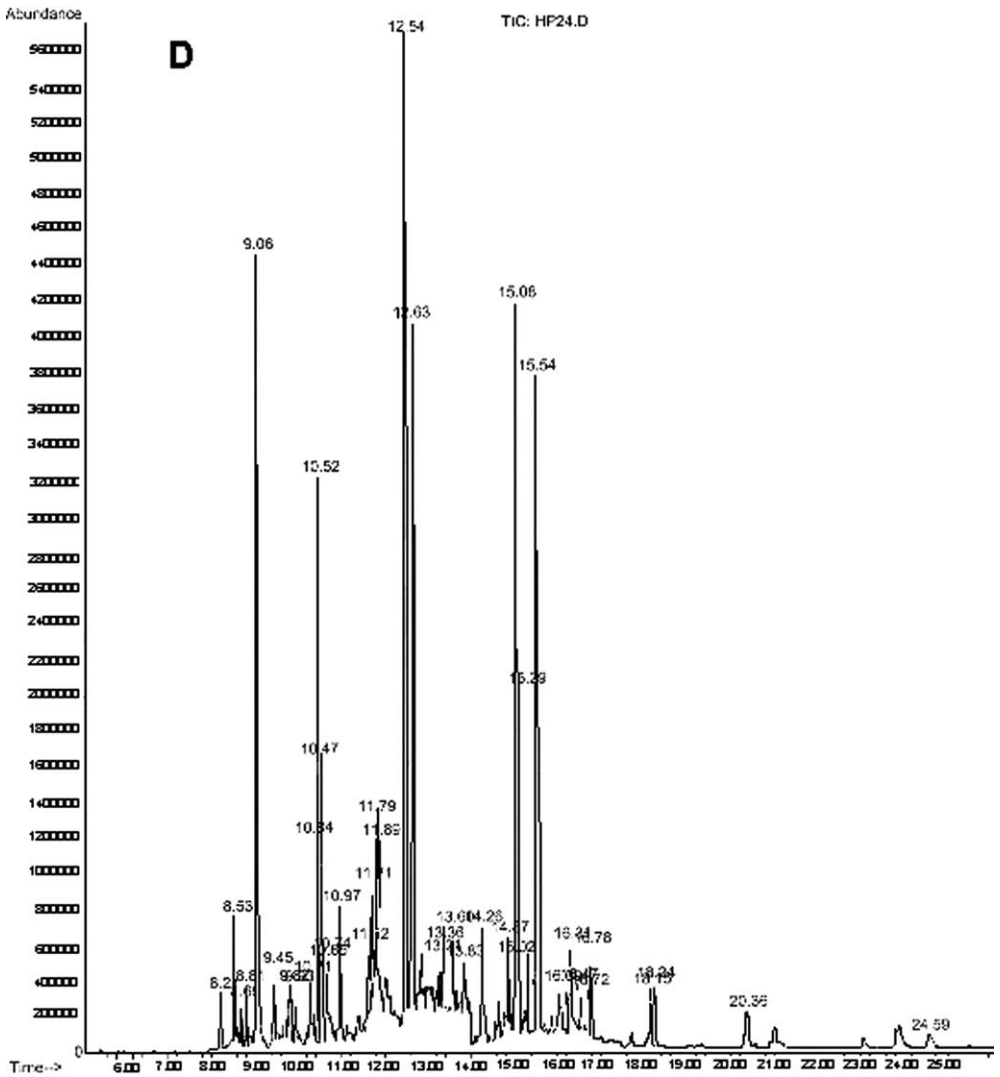


Fig. 3 (*continued*).

levels in smoked shellfish should be a cause for concern, particular in an area where the presences of DSP phycotoxins are endemic.

This study clearly shows that the consumption of smoked shellfishes in the way that are prepared in the southern Chile, is a source of exposure to PAHs and diarrhetic phycotoxins together. Since there is a consensus on the substantial contribution of PAHs to cancer in humans, with the addition of DTX-1, a strong tumor promoter, the population is in high risk to develop digestive cancer. Remedial actions and monitoring programs of these smoked shellfish must be implemented. It is also important in assessing the effects of smoked shellfishes consumption in epidemiologic studies in the population of the Patagonia fjords witch normally consume shellfish prepared in this manner. Environmental carcinogen exposure may play an important role in the incidence of cancer in population. In addition to environmental pollutants the intake of food smoked may be a contributing factor.

The DSP illness has a worldwide distribution but is most prevalent in Europe, Japan and Chile, where shellfish culture and salmon aquaculture are extensively carried out. The accurate measurement of DSP phycotoxins is a topic of growing concern worldwide and it should be in Chile, since its three most austral Regions have endemic DSP contamination since 1970 (Avaria, 1979; Lembeye and Yasumoto, 1993; Zhao et al., 1993; Uribe et al., 2001).

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