CIENCIA 4(4), 335-342, 1996
Maracaibo, Venezuela

Study on DSP and PSP toxic profile in *Haliotis tuberculata*

A. Gago-Martínez, M. Comesaña-Losada, J.M. Leao-Martins and J.A. Rodríguez-Vázquez*

Dpto. de Química Analítica y Alimentaria, Facultad de Ciencias, Universidad de Vigo Apartado 874, 36200. Vigo, Spain

Recibido: 23-09-96 Aceptado: 25-11-96

Abstract

Galician coastal waters (NW of Spain) present the suitable conditions for the growth of a great amount of different species, overall the bivalve molluscs. However, the presence of marine biotoxins during some periods of the year seriously affects the Galician industry, causing important socioeconomic damage. The abalone, *Haliotis tuberculata*, is a species that it was began to exploit since time ago. This species is exported to Japan where it is very appreciated. The abalones are also affected by the appearance of several species of dinoflagellates in marine phytoplankton which produce paralytic (PSP) and diarrhetic toxins (DSP). Bioassay with mouse is the official method for the analysis of these toxic compounds but due to its lack of sensitivity, High Performance Liquid Chromatography with fluorescent detection (HPLC-FLD) was proposed as an analytical alternative because of its sensibility and selectivity for these toxic components. In this study we report on the work carried out by applying HPLC-FLD methods for the analysis of marine biotoxins present on the studied samples.

Keywords: Abalone; diarrhetic and paralyzing shellfish poisonings; fluorescence detection; *Haliotis tuberculata;* liquid chromatography; toxins.

Estudio del perfil tóxico diarreico y paralizante en la oreja de mar, *Haliotis tuberculata*

Resumen

Las aguas de las costas gallegas (NW España) presentan las condiciones adecuadas para que en ellas se desarrollen gran cantidad de especies, sobre todo los moluscos bivalvos. Sin embargo, la presencia de biotoxinas marinas en determinadas épocas del año afecta notablemente a estas costas, causando graves daños a nivel socioeconómico. Una especie que se ha comenzado a explotar desde hace algún tiempo es la oreja de mar, *Haliotis tuberculata*, la cual se exporta sobre todo a Japón, donde es muy apreciada. Debido a la repetida ocurrencia de episodios de contaminación del plancton marino, por la presencia de distintas especies de dinoflagelados tóxicos productores, entre otras, de toxinas paralizantes (Paralytic Shellfish Poisoning-PSP) y diarreicas (Diarrhetic Shellfish Poisoning-DSP), fue necesario el planteamiento de métodos de análisis para su control. El bioensayo con ratones es el método oficial de análisis de estos compuestos tóxicos en la mayoría de los países; sin embargo, debido

* To whom correspondence should be addressed.

a la falta de sensibilidad y selectividad del mismo, fue necesaria la búsqueda de alternativas metodológicas más sensibles. La Cromatografía Líquida de Alta Eficacia surgió como punto importante de partida para paliar la problemática del ensayo biológico. En este estudio se muestran los resultados obtenidos mediante la aplicación de los métodos de HPLC-FLD para el análisis de biotoxinas marinas presentes en las muestras de *Haliotis tuberculata* objeto de nuestro estudio.

Key words: Cromatografía líquida; detección de fluorescencia; *Haliotis tuberculata*; intoxicación diarreica y paralizante; toxinas.

Introduction

The frequent appearance of toxic blooms in Galician waters is a relevant phenomenon with important socio-economic implications in this area, where shellfish industry is one of the main economic activities. These toxic events, commonly known as "red tides", are produced by the proliferation of some unicellular phytoplanktonic toxic species, called dinoflagellates, which reproduce in big amounts in some periods of the year.

Paralytic Shellfish Poisoning (PSP) and Diarrhetic Shellfish Poisoning (DSP) are the main effects of contaminations in Galician sea waters caused by marine biotoxins. Recently, Amnesic Shellfish Poisoning toxins (ASP) were also found as another contaminant in this area (1). Due to the relevance of these contaminations, from a socioeconomic point of view, several studies were focused to the determination of these toxic compounds. The chemical structures of both DSP and PSP toxins are shown en Figure 1 (a,b).

Bivalve molluscs are the most affected species during the toxic blooms due to its characteristic filtration feeding. Due to the socioeconomic implications, originated by their contamination, its sanitary control (2) is well justified. The presence of PSP and DSP toxic compounds has recently been studied in the abalone *Haliotis tuberculata* (3) which posses a high potential for toxin accumulation. This species is a marine gastropod from the genus *Haliotis*, distributed along the coasts of most of the European continent and islands bathed by the Pacific, Indian and Atlantic oceans (4); however, this type of gastropod mollusc is not widely exploited in Galicia, being exported, most of its production, to Japan.

In early spring of 1994 took place an important toxic event at Ria de Vigo, which caused the contamination of several species of molluscs. Some samples of *Haliotis tuberculata* were analyzed for these toxic compounds, being the aim of this work the study of the toxic profile of these species contaminated during this toxic event.

Materials and Methods

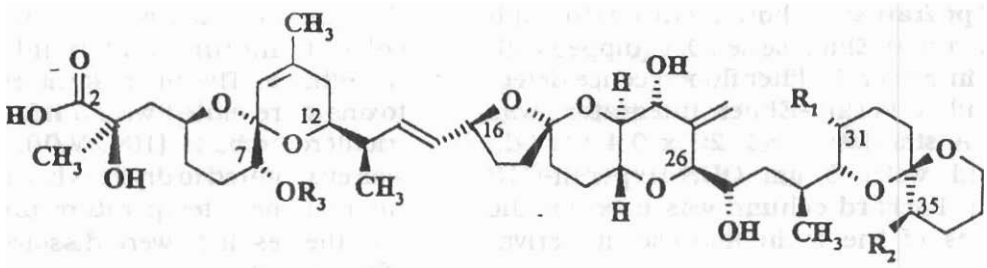
Abalones from Galician Rías (Ría de Vigo, NW of Spain) were collected in March 1994 and kindly provided by Viva Galicia S.A. Whole tissue of the abalones was homogenized and frozen until their DSP and PSP analyses were carried out.

DSP Toxin Analysis

Standards: Standards of Okadaic acid were kindly provided by Dr. M.A. Quilliam from the Institute for Marine Biosciences (NRC), Halifax, Canada.

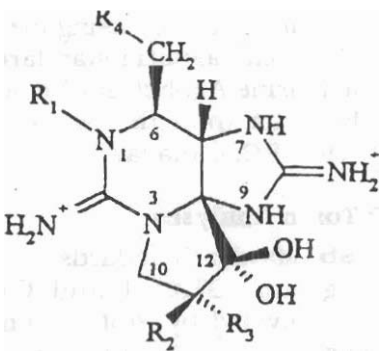
Reagents: 9-Anthryldiazomethane was synthesized in house according to Yoshida *et al.* (5), with further modifications (6). N-chlorosuccinimide and quinuclidine were purchased from Sigma Chem Co. and Aldrich Chem. Co., respectively. Analytical grade solvents (tetrahydrofurane (THF), n-hexane, acetonitrile, methanol, trichlorometane) were purchased from Panreac.

a)



R_1	R_2	R_3	
CH_3	H	H	Okadaic acid (OA)
CH_3	CH_3	H	Dinophysistoxin-1 (DTX1)
H	CH_3	H	Dinophysistoxin-2 (DTX2)
H or CH_3		Acyl	Dinophysistoxin-3 (DTX3)

b)



STX = saxitoxin
NEO = neosaxitoxin
GTX = gonyautoxins

R_1	R_2	R_3	R_4	Carbamate toxins	N-Sulfocarbamoyl toxins	Decarbamoyl toxins
				$-O-\overset{O}{\overset{\|}{C}}-NH_2$	$-O-\overset{O}{\overset{\|}{C}}-NHSO_3^-$	$-OH$
H	H	H		1 (STX)	2 (GTX5, B1)	3 (dcSTX)
H	H	OSO_3^-		4 (GTX2)	5 (C1)	6 (dcGTX2)
H	OSO_3^-	H		7 (GTX3)	8 (C2)	9 (dcGTX3)
OH	H	H		10 (NEO)	11 (GTX6, B2)	12 (dcNEO)
OH	H	OSO_3^-		13 (GTX1)	14 (C3)	15 (dcGTX1)
OH	OSO_3^-	H		16 (GTX4)	17 (C4)	18 (dcGTX4)

Figure 1. Chemical structure of DSP toxins (a) and PSP toxins (b).

Apparatus: A liquid chromatograph model Perkin-Elmer serie 10, equipped with a Perkin-Elmer-10 filter fluorescence detector and a Perkin-Elmer integrator, was used. A stainless steel, 25 x 0.4 cm i.d., packed with 5 µm ODS-Hypersil-C18 Hewlett-Packard column was used for the analyses of the toxin fluorescent derivatives.

Extraction of DSP toxins: 2 g of whole tissue of abalones were homogenized with 10 mL methanol 80% for 1 min. The sample was centrifuged and the supernatant was decanted, washed with hexane, and partitioned into chloroform as described by Quilliam (7). The combined chloroform extracts were evaporated to dryness in a rotary evaporator at room temperature under vacuum and then the ressidue was dissolved in 1 mL of methanol.

Preparation of 9-anthryldiazomethane (ADAM): ADAM was synthetized in situ under the following conditions based on the improvements proposed by Quilliam *et al.* (6). To 500 µL of 35 mmol/L 9-anthraldehyde hydrazone in (THF), 500 µL of 69 mmol/L quinuclidine solution in THF and 500 µL of 35 mmol/L N-chlorosuccinimide in THF were added. The solution was mixed and kept at room temperature for 1 hour in dark, and the obtained reagent was immediately used for derivatization.

Derivatization reaction: Aliquots of 35 µL of the methanol extracts of DSP were evaporated to dryness under gentle stream of nitrogen at room temperature and then, the DSP were derivatized with 100 µL of ADAM solution prepared as above described. After 2 hours of reaction in dark at 37°C, the obtained fluorescent derivatives were subsequently cleaned up prior LC-FLD analysis.

Cleanup: After evaporating the solvent under N_2, the reaction products were purified by transferring them to a Sep-pak silica cartridge (Waters, Millipore), with 1 mL of hexane-trichloromethane solution (50% V/V) and washed with 5 mL of the same solvent mixture and 5 mL of trichloromethane. The fluorescent esters of DSP toxins were eluted with 5 mL of methanol-trichloromethane (10% V/V). The eluated was evaporated to dryness in a rotary evaporator at room temperature under vacuum and the residues were dissolved in 500 µL of methanol.

DSP toxin analysis by LC-FLD: Derivatized samples were analyzed by liquid chromatography, using the HPLC system described above, under isocratic conditions using acetonitrile-water (85% V/V) as the mobile phase. The flow-rate was 1 mL/min and the injection volume 10 µL. The detection was carried out with excitation wavelength at 365 nm and emission wavelenght at 412 nm. Calibration studies were previously carried out by using methanolic solutions of okadaic acid standards provided by the Marine Analytical Chemistry Standard Program from the National Research Council, NRC, Canada.

PSP Toxin Analyses

Standards: Standards of PSP toxins (STX group, GTX1-4 and C1-C4) were kindly provided by Prof. Oshima, Tohoku University, Japan. Standards of STX, neoSTX and GTX2/3 were also provided by Dr. Pierre Thibault, Institute for Marine Biosciences, NRC, Canada.

Reagents: Periodic acid, sodium phosphate, ammonium phosphate (Probus), tetrabutylammonium phosphate, 1-heptane sulfonic acid (Sigma Chem Co.), acetic acid and HPLC grade acetonitrile (Panreac) were used.

Apparatus: A liquid chromatograph model Perkin-Elmer LC-10, equipped with a post-column derivatization system, a F-1000 double monocromation Hitachi fluorescence detector and a Hewlett-Packard 3390-A integrator was used. The post-column derivatization system was composed by a 10 m x 0.5 mm i.d. teflon

introduced in a thermostatic water bath at 65°C and two driving bombs (for the oxidizing reagent at a flow rate of 0.6 mL/min and for the acid solution at a flow rate of 0.4 mL/min). A stainless steel, 25 x 0.4 cm i.d., packed with 5 μm Develosyl-C8 Nomura Chemical column was used for the analyses of the toxin fluorescent derivatives.

Extraction of PSP toxins: 25 g of whole abalone tissue were homogenized with 25 mL 0.1 M Hydrochloric acid. Some portions of the homogenate were centrifuged and 1 mL of the supernatant was ultrafiltered (Millipore Ultrafree-MC) and analyzed directly by liquid chromatography with fluorescent detection.

PSP Toxin Analysis by LC-FLD: Chromatographic separation was performed by using ion-pair chromatography based on the method described by Oshima (8) with some slight modifications, using 7 mM periodic acid in 50 mM sodium phosphate at pH 9.0 as the oxidizing reagent. Three different mobile phases were used in order to analyze the three different groups of PSP compounds with a flow of 0.8 mL/min:

a) 2 mM Tetrabutylammonium phosphate adjusted to pH 5.8 with acetic acid 0.05 N (for epi GTX8, GTX8, C3 and C4).

b) 2 mM 1-heptane sulfonic acid in 10 mM ammonium phosphate at pH 7.3 (for GTX1-6).

c) 2 mM 1-heptane sulfonic acid and 30 mM ammonium phosphate (pH 7.1) in 5% v/v acetonitrile (for STX, neoSTX and dcSTX).

The toxic compounds were analyzed with the HPLC system described before and the fluorescence detector was set at 330 nm excitation and 390 nm emission.

Results and Discussion

DSP

The results of the analyses carried out in the Galician samples of *Haliotis tuberculata* are shown in Figure 2b. This chromatogram shows a peak at retention time of 11 min. which corresponds with okadaic acid (OA), as shows the OA standard in Figure 2a.

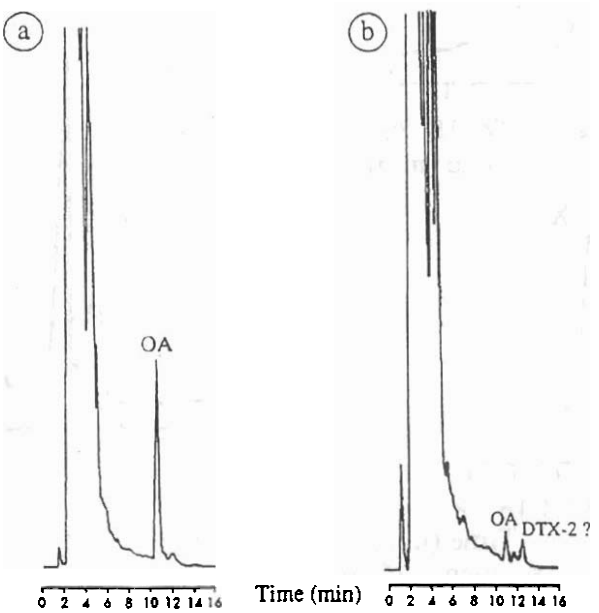


Figure 2. a) Standard of OA and b) Diarrhetic toxic profile of Galician abalone sample.

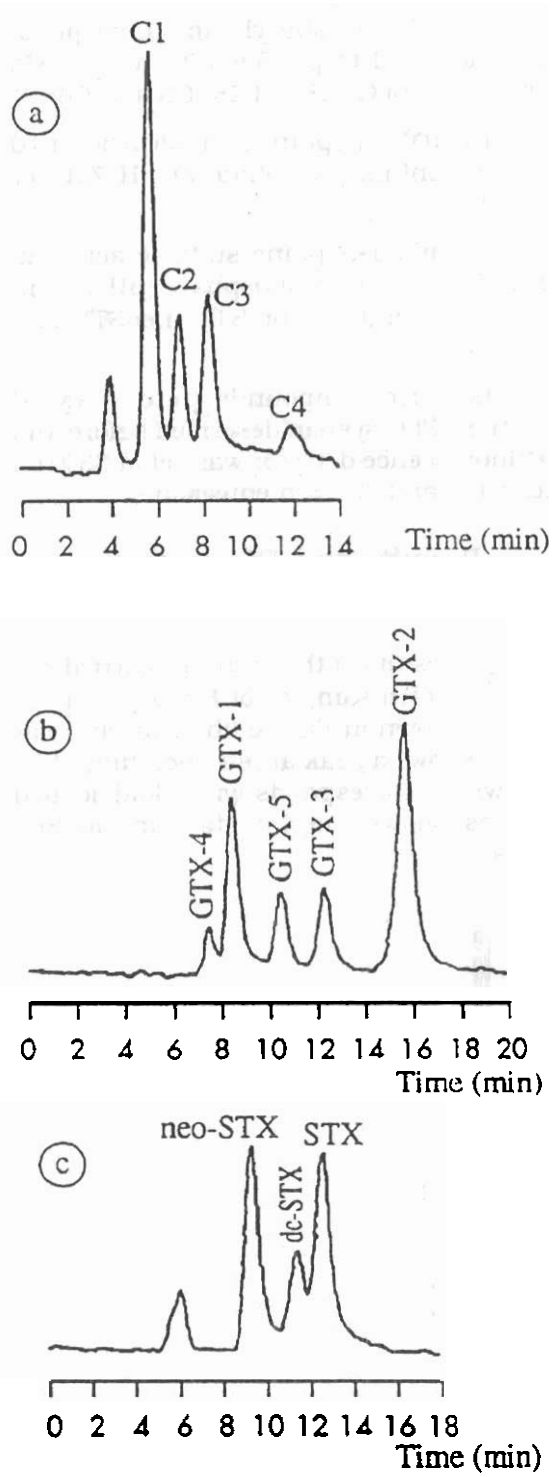


Figure 3. PSP toxin standard solutions: a) C toxin group, b) GTX group and c) STX group.

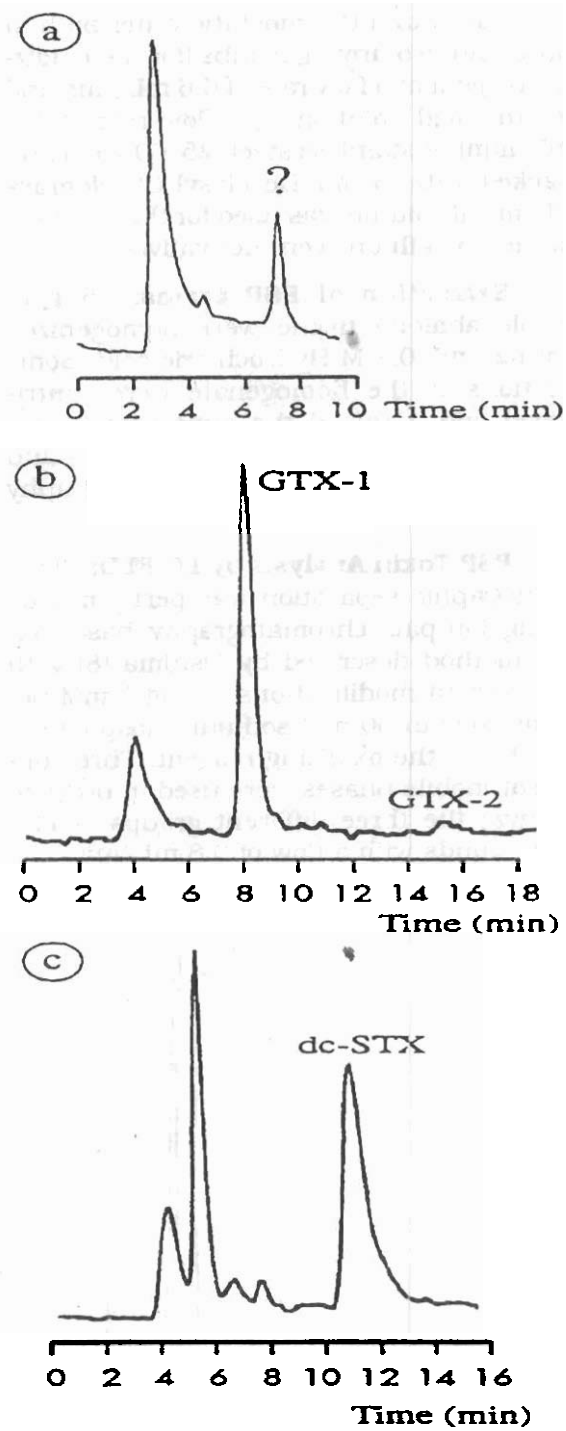


Figure 4. Paralyzing toxic profile of Galician abalone sample from Ría de Vigo: a) C toxin group profile, b) GTX group profile and c) STX group profile.

The chromatogram shows the presence of another peak close to OA, which could be associated with an isomer of okadaic acid, DTX2. Due to the unavailability of standards of this new compound, this hypothesis requires further confirmation by using more sensitive analytical techniques such as liquid chromatography-mass spectrometry (LC-MS), which due to the ion fragmentation allows to obtain information about the presence of this compound. Quantitative results for OA were obtained by using calibration solutions of this toxin from the NRC (Marine Standards Program). As a result of this quantitation the mean amount of OA found in these samples was 0.95 µg/g of whole abalone tissue.

PSP

The analysis for C toxins was not successfully carried out (Figure 4a). Three well defined peaks were obtained at retention times which do not match exactly with those corresponding to the standard (Figure 3a).

The analysis of STX group allows to conclude that dc-STX is the predominant compound in the studied samples, as shown in Figure 4b. The quantitation of this component revealed a concentration of 3.9 mg eq. STX/Kg of tissue.

The toxic profile obtained for GTX group is shown in Figure 4c. This profile is not easily explainable due to the presence of compounds, whose retention times did not match exactly with those obtained for the standards. GTX1 and GTX2 were identified as the predominant compounds of this group. Quantitative results were obtained by using a calibration solution of GTX toxins, kindly provided by Prof. Oshima and Dr. Pierre Thibault. The results of this quantitation revealed that 0.9 mg eq. and 0.01 mg eq. STX/Kg of tissue, respectively, were present.

Previous studies carried out in abalone samples (9,10) agree with the presence of dc-STX as prioritary compound from the STX group. The presence of toxins from GTX group and C group is an unfrequent profile but a larger proportion of C1, C2 and gonyautoxins have also been found in viscera of some specimens of abalone (11).

Conclusions

An unusual toxic profile was found for abalone samples previously exposed to a red tide. PSP toxins were present in the studied samples as well as small amounts of OA. The possible presence of DTX2 was detected as well. Concerning to PSP toxins, dcSTX is the main PSP component of the STX group present in these samples and GTX1 and GTX2 were the major GTX's compounds found.

Acknowledgments

Authors thank M. Quilliam from the Institute for Marine Biosciences, NRC, Halifax (Canada) and Oshima from Tohoku University, Sendai (Japan) for providing standards of Okadaic acid and PSP toxins, respectively. Standards of some PSP compounds were also kindly provided by P. Thibault from the Institute for Marine Biosciences, NRC, Halifax, Canada. Abalone samples from Ria de Vigo provided by "Viva Galicia S.A.", are acknowledged as well.

J.M. Leao acknowledges the predoctoral support of JNICT in the Praxis Program.

References

1. MÍGUEZ A., FERNÁNDEZ M.L., FRAGA S. First detection of domoic acid in Galicia (NW Spain). ***Seventh International Conference on Toxic Phytoplankton***, Sendai (Japan), 1995 (in press).
2. YASUMOTO T., OSHIMA Y., YAMAGUCHI M. ***Bull Jpn Soc Sci Fish*** 44: 1249, 1978.
3. MARTÍNEZ A., FRANCO J.M., BRAVO I., MAZOY M., CACHO E. ***Toxic Phytoplankton Blooms in the Sea***. T.J. Smayda and

Y. Shimizu, (eds.) Elsevier pub., pp. 419-423, 1993.

4. BROWN L.D. ***J Molluscan Studies*** 54 (4): 429-443, 1993.
5. YOSHIDA T., UETAKE A., MURAYAMA H., NIMURA H., KINOSHITA T. ***J Chrom*** 348: 425-429, 1985.
6. QUILLIAM M.A., HARDSTAFF W.R., GAGO-MARTÍNEZ A., RODRÍGUEZ-VÁZQUEZ J.A. (manuscript in prep.).
7. QUILLIAM M.A. ***J AOAC Int*** 78 (2): 555-570, 1995.
8. OSHIMA Y., MACHIDA M., SASAKI K., TAMAOKI Y., YASUMOTO T. ***Agric Biol Chem*** 48 (7): 1707-11, 1984.
9. BRAVO I., CACHO E., FRANCO J.M., MÍGUEZ A., REYERO M.I., MARTÍNEZ A. Study of PSP toxicity in *Haliotis tuberculata* from Galician coast. ***Seventh International Conference on Toxic Phytoplankton***, Sendai (Japan), 1995 (in press).
10. NAGASHIMA Y., ARAKAWA O., SHIOMI K., NOGUCHI T. ***J Food Hygien Soc Jap*** 35 (5): 548-549, 1994.
11. LIN H.S., KAMOTA M., FUJIWARA N., OSHIMA Y., YASUMOTO T. Paralytic shellfish toxins in the abalone, *Haliotis tuberculata*, imported from Spain. ***Seventh International Conference on Toxic Phytoplankton***, Sendai (Japan), 1995 (in press).