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Toxicity and toxin identification in *Colomesus asellus*, an Amazonian (Brazil) freshwater puffer fish

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Abstract

Toxicity and toxin identification in *Colomesus asellus*, an Amazonian (Brazil) freshwater puffer fish. By using four different techniques—mouse bioassay, ELISA, HPLC and mass spectrometry—we evaluated the toxicity in the extracts of *C. asellus*, a freshwater puffer fish from the rivers of the Amazon, and identified for the first time the components responsible for its toxicity. The T20G10 monoclonal antibody raised against TTX, and employed in an indirect competitive enzyme immunoassay, showed very low affinity for the *C. asellus* extracts, indicating that TTX and its analogs are not the main toxic components of the extracts. This antibody was efficient in detecting presence of TTX in a total extract of *Sphoeroides spengleri,* which is one of the most toxic puffer fish found in the Atlantic coast. Extracts of *C. asellus* were toxic when administered intraperitonially into mice with an average toxicity of 38.6 ± 12 mouse unit (MU)/g, while HPLC analysis indicated a lower toxin content (7.6 ± 0 5 MU/g). The HPLC profile showed no traces of TTX, but only the presence of PSPs (STX, GTX 2 and GTX 3). These toxins were also confirmed by electrospray ionization mass spectrometry.
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1. Introduction

Puffer fishes are poisonous and may cause a characteristic clinical poisoning with a high mortality rate, but in Japan and China, have long been a delicacy (Lange, 1990). In Occidental countries, puffer fishes are only sporadically consumed and have been involved in several human poisonings, mainly by the species belonging to the genus *Sphoeroides* (Almeida and Rocha, 1989; Ochoa et al., 1997; MMWR, 2002). Toxinological studies of some *Sphoeroides* species have been reported (Correa et al., 1990; Freitas et al., 2003; Oliveira et al., 2003).

The poison of freshwater puffers may be composed by tetrodotoxin (TTX) or saxitoxin (STX) and its analogs, the predominant toxin being

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dependent on the species. These guanidinium toxins inhibit electrical signaling in many excitable cells (nerves and muscles) by binding to the site 1, blocking the pore of the voltage-gated sodium channels (VGSC) (Cestèle and Catterall, 2000).

The correct identification whether which toxin, TTX or STX, is the main toxic component of a determined puffer poison is not always a simple task. For example, *Tetraodon fangi*, the Thailand freshwater puffer involved in some food poisonings in humans (Saitanu et al., 1991), was first reported to posses mainly TTX as its poison (Laobhripatr et al., 1990). Later, however, the major toxins of this fish were identified as STXs (Sato et al., 1997). Furthermore, other freshwater puffers such as *Tetraodon leiurus complex* and *Tetraodon suvatii* from Thailand and *Tetraodon cutcutia* and *Chelonodon patoca* from Bangladesh may posses many paralytic shellfish poisoning (PSP) toxins (such as STX, neo-STX, decarbamoyl-STX, GTX 2, GTX 3, and decarbamoyl-GTX 2 and 3) (Kungsuwan et al., 1997; Zaman et al., 1997).

Nowadays, at least 185 species of puffer fishes in the family Tetraodontidae, distributed in 28 genera are known. In the genus *Colomesus* there are only two species described (Fishbase, 2005). Both species occur in Brazil: *Colomesus asellus* (Amazon puffer) is an exclusively freshwater puffer living in the Amazon basin, and *Colomesus psittacus* (banded puffer) inhabits brackish waters at the Brazilian northeast coast estuaries. *C. psittacus* was reported to possess an edible flesh and a very toxic liver (Sawaya, 1966), but the toxins and toxicity of *C. asellus* are as yet completely unknown.

In this work, by using four different techniques (mouse bioassay, ELISA, HPLC and mass spectrometry), we report for the first time the high toxicity found in *C. asellus* and the identification of PSPs (STX and gonyautoxins) as the main components of its poison.

2. Material and methods

2.1. Extraction

Twenty-four specimens of *C. asellus* (Müller and Troschel, 1848) were collected from the River Tocantins, in the town harbor of Cametá, Pará State, Brazil, in March of 2003. The total length of animals (in cm) and body weight (in grams) varied from 9 to 15.6 and from 18 to 96, respectively. The extraction procedure was according to Oliveira et al. (2003) with slight modifications. Briefly, six separated extracts were prepared from total body of *C. asellus* as described below (for the number of specimens used and weights, see Table 1).

Extracts were prepared by double extraction with 1% acid acetic in 70% ethanol (2 ml/g), filtered,

Table 1
Toxicity of *Colomesus asellus* extracts (MU means mouse unit) evaluated by different techniques

Sample	Number of specimens	Average body weight (g)	Toxicity				ELISA[a]
			Mouse bioassay		HPLC		
			MU/g	MU/indiv	MU/g	MU/indiv	
1	4	172.00	53.2	2287.5	17.74	764.5	0.0045
2	4	162.60	31.2	1268.3	4.99	202.8	0.0057
3	3	82.60	46.0	1267.5	10.18	280.3	0.0022
4	6	176.80	44.0	1296.7	5.35	157.6	0.0032
5	3	212.20	37.8	2675.0	4.36	308.4	0.0027
6	4	121.50	19.4	590.20	3.23	98.1	0.0064
			38.60±12.01	1564.20±768.49	7.64±5.49	301.95±239.45	0.0041±0.0017
Positive control[b]	285	10.92	45.53	nd	nd	nd	93.77

Six independent total body extracts of *C. asellus* were analyzed by mouse bioassay, high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA), and the toxicity expressed as mouse unit per gram of fresh tissue (MU/g) or per mean of body weight from one specimen individually (MU/indiv). A total body extract of several specimens of *Sphoeroides spengleri* was employed as positive control.

[a]Except to the *S. spengleri* extract that was based on it's IC_{50} (converted to MU/g of fresh tissue), the values were obtained from the highest concentration of each extract.

[b]Extract of *Sphoeroides spengleri* (positive control); nd, not determined.

defatted with 80% methylene chloride, vacuum concentrated, and ressuspended with distilled water to a known concentration.

2.2. Toxicity assay

The toxicity of *C. asellus* extracts was initially determined in accordance with the method of Kawabata (1978). After performing preliminary analysis by high performance liquid chromatography (HPLC), it was found that the toxic compounds were PSPs and, in addition, a mouse bioassay according to the AOAC method was carried out (Williams, 1984).

2.3. ELISA assay

In order to verify the possible presence of TTX in the extracts of C. *asellus* we carried out an enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody raised against TTX (MAb-TTX). The assay was according to Raybould et al. (1992).

2.4. Preparation of a conjugate-coat antigen

A conjugate-coating antigen (BSA-TTXF) was prepared by mixing bovine serum albumin (89.5 μl of BSA at 33.6 mg/ml), TTX (350 μl at 1 mg/ml) and formaldehyde (20.5 μl of a 37% solution, added drop wise). The reaction mixture was incubated under shaking at 37 °C for 3 days, and subsequently dialyzed against four changes (of 1 L) of 50 mM sodium phosphate, pH 7.0, containing 150 mM NaCl (PBS) at 4 °C. The conjugate concentration was determined spectrophotometrically using an extinction coefficient at 280 nm of 0.667 mg.cm/ml.

2.5. Indirect competitive inhibition enzyme immunoassay (ICEIA) for TTX

An immulon microtiter plate (Dynatech Laboratories Inc., Chantilly, VA) was coated with 100 μl/well of BSA-TTXF at a concentration of 5 μg/ml in PBS, and kept under incubation at 4 °C overnight. This plate was then washed three times with PBS containing 0.05% (v/v) tween-20 (PBS-T) and blocked with 200 μl/well of blocker solution (PBS-B: 0.5% gelatine (w/v) + 0.2% tween (v/v) in PBS) for 2 h at room temperature. Separately, plastic tubes were filled with 75 μl of each sample: (a) serial dilutions from the highest concentrations of each extract of *C. asellus*; (b) TTX standard curve as a control; and (c) an extract from *Sphoeroides spengleri*, a marine puffer fish that possesses high amounts of TTX (Oliveira et al., 2003), employed as a positive control extract for the presence of TTX. These samples were challenged with 75 μg/ml of Mab-TTX (final fixed concentration per tube for all experiments = 100 ng/ml); the highest standard TTX concentration used was 2.5 μg/ml. After 2 h incubation the contents of the tubes (100 μl) were transferred to the previously blocked plate. This plate was incubated at room temperature for 1 h and then washed three times with PBS-T. Peroxidase-rabbit anti-mouse IgG (H + L), diluted in PBS was added, and after incubation for 1 h at room temperature, the plate was again washed out and 100 μl of ABTS added, followed by a new incubation for 15 min. The reaction was stopped with 25 μl hydrofluoric acid (50%, v/v) and the absorbance at 405 nm measured in a microtiter plate reader Spectra MAX 250 (Molecular Devices, CA, USA). Six independent experiments, in duplicate, were performed for all samples. Fifty percent inhibitory concentration (IC_{50}) and values from TTX standard curve, expressed in μg/ml, were used to establish the sensitivity of the method and to estimate the different toxin concentrations.

Monoclonal antibody T20G10 against TTX (MAb-TTX) was from Hawaii Biotechnology Group, Hawaii. Peroxidase-rabbit anti-mouse IgG (H + L) was from Zymed, USA, 2,2′-azino-di (3-ethylbenzothiazoline-6-sulfonate) (ABTS) from Boehringer Mannheim, Germany and hydrofluoric acid from Sigma, USA.

2.6. References toxins

Tetrodotoxin was obtained from Sankyo Co., Japan. Saxitoxin was obtained from US Food and Drug Administration, USA, and Gonyautoxins were a kind gift from Dr. Takehiko Ogata, Kitasato University, Japan.

2.7. Purification of STX and analogs

The toxic extracts were partially purified by ion-exchange chromatography using an Amberlite GC-50 NH_4^+ (1.0 × 6.0 cm) column. The retained material was eluted with 10% acetic acid, vacuum concentrated, and submitted to treatment with active charcoal (Norit-A). The adsorbed toxins were eluted with 1% acetic acid in 20% ethanol

(Goto et al., 1965), and the solvent subsequently evaporated to dryness at 65 °C. The dried material was diluted in milli-Q water, filtered in a 0.22 μm membrane and further submitted to HPLC.

2.8. HPLC-FLD analysis

TTXs were detected by HPLC carried out on a Shimadzu LC-10A HPLC system according to Pires et al. (2002). A Shimpack C_{18} column (4.6 × 250 mm i.d., Shimadzu Corp., Japan) was used with a mobile phase of 60 mM heptanesulfonic acid and 10 mM ammonium phosphate buffer (pH 5.0). The eluate from the column was continuously mixed with an equal volume of 4 M NaOH and heated in a reaction coil at 110 °C. The toxin was detected with a fluorescence detector, monitoring the fluorescence at 500 nm with 380 nm excitation.

PSP analysis was carried out in the same HPLC system, as described previously (Oshima, 1995). A Synergy-Fusion C_{18} Phenomenex column (4.6 × 150 mm i.d., GenTech Scientific Inc., NY, USA) was used in combination with the two mobile phases: (I) 2 mM heptanesulfonic acid in 10 mM ammonium phosphate buffer (pH 7.1) for gonyautoxins (GTXs), and (II) 2 mM heptanesulfonic acid with 3% acetonitrile, in 30 mM ammonium phosphate buffer (pH 7.1) for STXs. The eluate from the column was continuously mixed with 7 mM periodic acid in potassium phosphate buffer (pH 9.0), and heated at 85 °C. The fluorophors formed were monitored at 390 nm with a 330 nm excitation.

2.9. Mass spectrometry analysis

Aliquots of the *C. asellus* extracts were analyzed by electrospray ionization in a Q-TOF Ultima API (Waters, Manchester, UK), operating in positive W mode. The sample was applied at a flow rate of 2 μl/min in a solvent mixture consisting of water/methanol/acetic acid (50:50:0.1). The capillary and cone voltages were set to 2.8 kV and 30 V, respectively.

3. Results and discussion

C. asellus is an Amazonian freshwater puffer fish that occasionally is sold by the aquarists. Apart from the popular knowledge that it is poisonous, this species is completely unknown to science, as there have been no studies concerning its biology, behavior, ecology and toxicity.

In this study, the toxicity of *C. asellus* extracts was initially evaluated by mouse bioassay. When injected into mice (i.p.) the toxicities varied from 19.4 to 53.2 mouse unit (MU)/g, as shown in Table 1. It has been reported that freshwater puffer fishes from rivers in Thailand may contain less than 50 MU/g in their tissues, as observed for *T. fangi* and *Tetraodon palembangensis* (Saitanu et al., 1991). On the other hand, species containing higher amounts of toxins are not rare. *T. leiurus* may exhibit toxicities varying from 35 to 750 MU/g depending on the source tissue, the ovary being the most toxic (Kodama and Ogata, 1984). Furthermore, likewise marine puffers, the toxicity of freshwater puffers may vary according to the location and the season in which the fish is caught (Kungsuwan et al., 1997). Here we present results only from total body extracts and, therefore, further studies will be necessary to evaluate the possible seasonality of the toxicity and the distribution of toxins in the tissues of *C. asellus*.

Since the toxin composition of the genus *Colomesus* is completely unknown, we performed the indirect competitive inhibition enzyme immunoassay (ICEIA) and HPLC analysis for TTXs, suspecting that the *C. asellus* could accumulate this toxin and its derivatives. Following this, analyses for PSPs were also performed.

The T20G10 MAb-TTX was employed in the ICEIA to detect TTX in the *C. asellus* whole body extracts. Competitive inhibition curves were obtained from a standard curve of TTX, an extract from *S. spengleri*, jointly with 6 extracts of *C. asellus,* as shown in Fig. 1(A and B). Linear regression of the standard curve was obtained, giving a correlation coefficient of 0.99339. These results demonstrated very high sensitivity that was confirmed by the lowest detection limit of TTX as close to 0.1 ng/ml (~0.00041 MU).

The highest concentration of the *C. asellus* extracts employed in the ICEIA, 15 mg/ml, was not able to completely inhibit the binding of all the content of MAb-TTX to the plate coated with BSAF-TTX. Based on this feature and since the highest concentration of standard TTX was only 2.5 μg/ml we could make, by this assay, only a rough estimation of the toxicity of the *C. asellus* extracts, expressing it as MU/g of fresh tissue (Table 1). For this, we used the highest concentration of each extract obtained from the partial curves (shown in Fig. 1), and the values did not represent the real toxicity in the extracts.

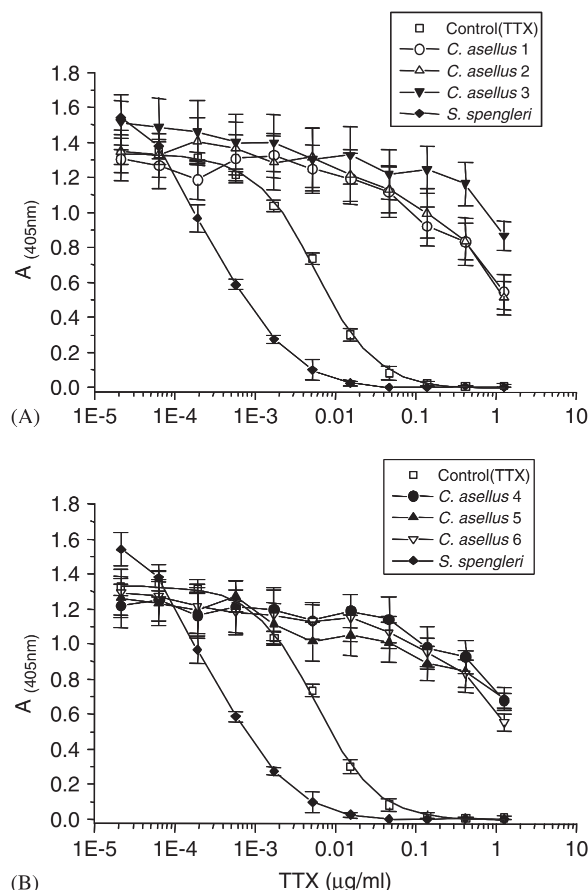

Fig. 1. Indirect CEIA using Mab-TTX T20G10 for the detection of TTX in puffer fish extracts. Six total extracts (1–3, A and 4–6, B) of *C. asellus*, diluted in PBS and a total extract of various *S. spengleri* specimens were compared with a TTX standard curve. The curves of TTX and *S. spengleri* are the same in (A) and (B); extracts from *C. asellus* were separated only to clear the visualization. IC_{50} of TTX and *S. spengleri* were 5.0 and 0.2 ng/ml, respectively. Vertical bars indicate the mean ±standard error of mean of six independent experiments, in duplicate.

The toxins in *C. asellus* extracts showed very low affinity in binding to the MAb-TTX, demonstrating that TTX is not the main toxic compound in these fishes. On the other hand, the *S. spengleri* extract, employed as a positive control, was able to completely inhibit the MAb-TTX binding to the BSA-TTX, resulting in an IC_{50} of 0.2 ng/ml. The toxicity curve of this extract shifted leftward, showing that this extract contains, at least, 125 ng/ml of TTX, which is 25-fold more than the TTX standard solution (5 ng/ml). This result could also suggest a possible cross-reaction with other TTX derivatives present in the *S. spengleri* extract. Raybould et al. (1992) reported that, despite the high specificity, the T2010 Mab-TTX might cross-react with other TTX analogs such as tetrodonic acid (TDA) and 4,9-anhydrotetrodotoxin (4,9-anhydroTTX), when these compounds are in high concentrations ($IC_{50} >$ 300 ng/ml). We reported that, besides TDA and 4,9-anhydroTTX, *S. spengleri* also contains 4-epiTTX (Oliveira et al., 2003), a compound not yet investigated in this aspect. As will be discussed later, using other techniques we were able to confirm that *C. asellus* does not contain TTX and its derivatives.

Many different ELISA methods based on monoclonal antibodies have been developed to detect TTX, diarrheic (dinophysistoxin-1 and okadaic acid) and PSP (STXs and GTXs) toxins (Chu and Fan, 1985; Núnez and Scoging, 1997; Kawatsu et al., 2002). These may be employed by local authorities to easily evaluate the toxicity of large numbers of samples. Our ELISA results confirmed the high specificity of the T20G10 against TTX, which enabled us to speculate that the cross-reaction of the *C. asellus* extracts, even in low levels, with MAb-TTX may be attributed to the high concentrations used in these assays.

The identification of STX and its derivatives in the *C. asellus* extracts was achieved by HPLC comparison of their retention time with that of the standard compounds. The HPLC-FLD analysis showed the presence of high quantities of STX, (Fig. 2(A and B)), and only traces of GTX 2 and GTX 3 (Fig. 2(C and D)). It has been reported that some species of puffer fishes may accumulate both STX and TTX, and sometimes only the association of more than one chemical technique, such as HPLC and mass spectrometry, may accurately discriminate these two toxins (Nakashima et al., 2004). In the case of *C. asellus*, there was no evidence for TTX and its derivatives by both HPLC and mass spectrometry (Data not shown). Therefore, our results clearly demonstrated that STX is the main toxic component in *C. asellus*. We also have determined that the STX-equivalent in all extracts was higher in the bioassay than the HPLC-FLD analysis. This might be due either to a synergistic effect with other PSPs that might contribute to the total toxicity observed in the mouse bioassay or to a partial recovery of the toxins in the HPLC analysis.

Considering the origin of the toxins, it is well known that puffer fishes do not produce TTX and their derivatives, instead the production is caused by various species of bacteria living in a symbiotic

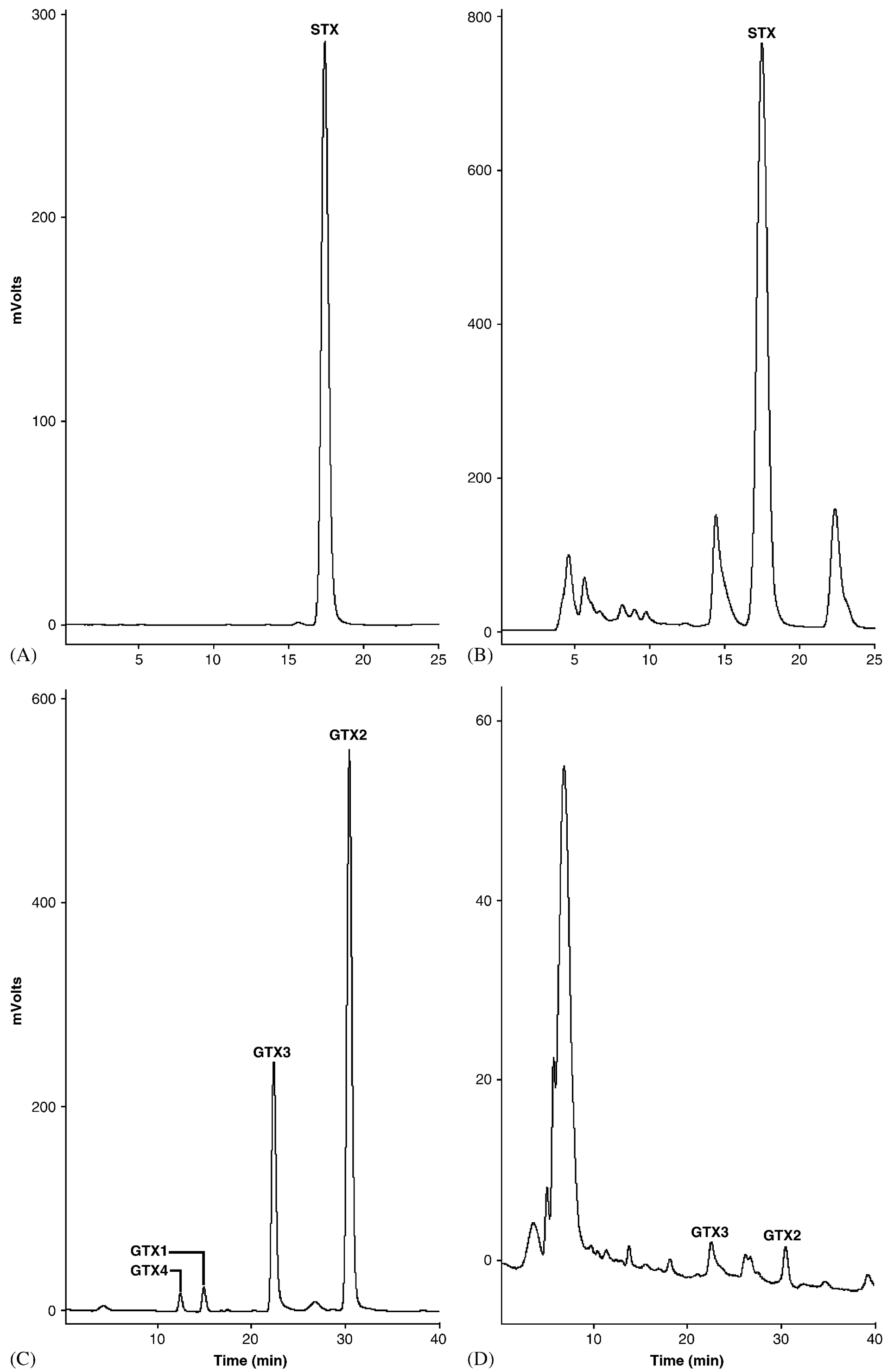

Fig. 2. Chromatogram profile in HPLC-FLD system of the standard toxins STX (A) and GTX 1–4 (C), and *Colomesus asellus* extract (B and D). Peak: saxitoxin (STX) and (GTX) gonyautoxin.

association with these fishes (Yotsu et al., 1987; Noguchi et al., 1987) or they are acquired through the food chain (Yasumoto and Yotsu-Yamashita, 1996). In the marine environment, STXs, which cause PSP, are produced by some species of dinoflagellates from the genus *Alexandrium* (*Gonyaulax*) (Schantz, 1986). It is also known that some xanthid crabs, horseshoe crabs and marine snails may posses PSP toxins, not associated with dinoflagellates (Sato et al., 1997). In freshwaters, certain species of cyanobacteria are involved in the production of these toxins (Lagos et al., 1999). Although we did not investigate the possible bacterial source of toxins present in *C. asellus* tissues, this cannot be ruled out, based on the fact that *C. asellus* is an exclusively freshwater puffer.

The presence of saxitoxin in the *C. asellus* extracts was confirmed by mass spectroscopy (Fig. 3). The spectrum showed the similar fragmentation pattern from m/z 300.2860 $(M+H)^+$ to those obtained by Fang et al. (2004) and Sleno et al. (2005). The daughter ions at m/z 282.1862, 240.0042, 196.1669, 182.7655, 161.8719 were consistent with the structures suggested by Sleno et al. (2005).

In conclusion, *C. asellus*, like other freshwater puffer fishes, accumulates saxitoxin, although no NeoSTX was present in our samples. In Brazil, some marine puffers are sporadically consumed by the local population (Oliveira et al., 2003), and poisoning cases were reported (Almeida and Rocha, 1989). In spite of there being no reports of food poisonings or even consumption of freshwater

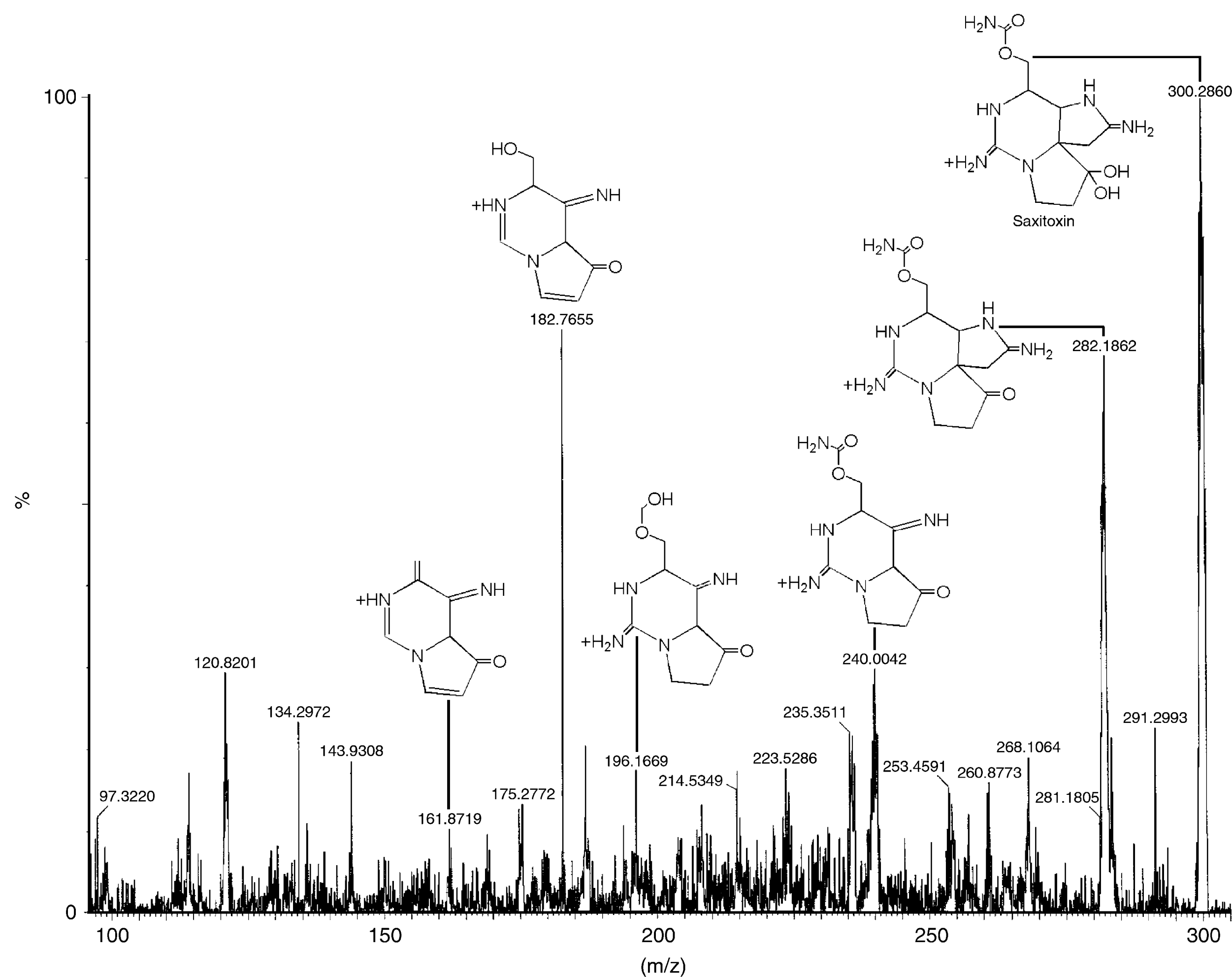

Fig. 3. Fragmentation ion profile of mass component in 300.2860 $(M+H)^+$ from the *C. asellus* extracts, showing a fragmentation pattern similar to STX obtained by Fang et al. (2004) and Sleno et al. (2005).

puffers by people in the Amazon region, we strongly recommend that the consumption of the Brazilian puffer *C. asellus* should be avoided.

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