Sains Malaysiana 43(7)(2014): 1003–1011

Distribution of Tetrodotoxin among Tissues of Pufferfish from Sabah and Sarawak Waters

(Taburan Tetrodotoksin antara Tisu di dalam Ikan Buntal dari Perairan Sabah dan Sarawak)

A. Mohd Nor Azman*, M. Samsur & M. Othman

ABSTRACT

*Puffer fish, mainly from Tetraodontidae family known to possess a neurotoxin or tetrodotoxin (TTX) which can cause a puffer fish poisoning and adverse effect to human health. In current study, the tetrodotoxin (TTX) concentration in different tissues (liver, skin, muscle) of 14 species of puffer fish from Sabah and Sarawak waters were analysed and determined by application of liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Overall, extracted toxin for all specimens were shown to be toxic with result more than 0.2 µg/g as calculated based on TTX standard curve. Among the tissues, liver were found to be highest in TTX concentration (91.0 µg/g), followed by muscle (51.1 µg/g) and skin (6.87 µg/g). Moreover, TTX concentrations among puffer fish species were significantly differences (*p<*0.05) with* Arothron immaculatus *(275 µg/g) showed highest mean value, while the lowest value was detected in* Lagocephalus lunaris *(4.92 µg/g). From this finding, LC-MS/MS application could be a potential tools to determine the TTX and advisedly used as a procedure in screening of seafood for monitoring program. Furthermore, baseline data of TTX levels in selected puffer fish from the study could be important information and used as guideline in order to mitigate puffer fish poisoning cases especially in East Malaysia waters.*

Keywords: East Malaysia; LC-MS/MS; puffer fish; Tetrodotoxin; tissues

ABSTRAK

*Ikan buntal, terutamanya daripada famili Tetraodontidae diketahui mengandungi neurotoksin atau tetrodotoxin (TTX) yang boleh menyebabkan keracunan ikan buntal dan memberi kesan buruk kepada kesihatan manusia. Dalam kajian ini, kepekatan tetrodotoxin (TTX) dalam tisu yang berbeza (hati, kulit, otot) dalam 14 spesies ikan buntal dari perairan Sabah dan Sarawak telah dianalisis dan ditentukan menggunakan spektrometri jisim kromatografi cecair/spektrometri jisim (LC-MS/MS). Secara keseluruhannya, toksin yang diekstrak daripada semua spesimen adalah toksik dengan keputusan melebihi daripada 0.2 µg/g berdasarkan pengiraan daripada keluk piawai TTX. Antara tisu yang dikaji, kepekatan TTX yang tertinggi didapati dalam hati (91.0 µg/g), diikuti oleh otot (51.1 µg/g) dan kulit (6.87 µg/g). Tambahan lagi, terdapat perbezaan ketara (*p<*0.05) dalam kepekatan TTX antara spesies ikan buntal dengan* Arothron immaculatus *(275 µg/g) menunjukkan nilai min tertinggi, manakala nilai terendah dikesan pada* Lagocephalus lunaris *(4.92 µg/g). Daripada penemuan ini, penggunaan LC-MS/MS boleh menjadi alat yang berpotensi untuk menentukan TTX dan boleh digunakan sebagai prosedur dalam pemeriksaan makanan laut bagi program pemantauan. Tambahan pula, data asas tahap TTX dalam ikan buntal yang dipilih daripada kajian boleh menjadi maklumat penting dan digunakan sebagai garis panduan dalam usaha untuk mengurangkan kes keracunan ikan buntal terutamanya di perairan Malaysia Timur.*

Kata kunci: Ikan buntal; LCMS/MS; Malaysia Timur; Tetrodotoksin; tisu

Introduction

Tetrodotoxin (TTX) is a non protein neurotoxin that is found in many diverse animals species such as puffer fish, some species of newts, frogs, gobies, flat worms, ribbon worms, starfish, crabs, the blue-ringed octopus and carnivorous gastropods (Miyazawa & Noguchi 2001; Mosher & Fuhrman 1984; Pires et al. 2002). Puffer fish were thought to accumulate TTX through the food chain, which starts from marine bacteria *Vibrio alginolyticus, Shewanella* sp., *S. putrefaciens, Alteromonas tetraodonis* and others (Matsui et al. 1990; Yasumoto et al. 1986). TTX intoxication from the ingestion of toxic puffers probably is the most common fish poisoning along the coasts of Asia. Outbreaks of puffer fish poisoning have been reported in various countries including Thailand (Brillantes et al. 2003; Laobhripatr et al. 1990), Mexico (Nunez-Vazquez et al. 2000), Hong Kong (Yu & Yu 2002), Australia (Isbister et al. 2002), Taiwan (Tsai et al. 2004), Bangladesh (Ahasan et al. 2004) and particularly Japan (Lin & Hwang 2001). In Malaysia, puffer fish poisoning has also been reported to occur in Sabah with one and nine death in 1985 and 1987, respectively (Kan et al. 1987; Lyn 1985). The latest case was reported in Johor with 34 cases due to ingesting puffer fish (Chua & Chew 2009).

Conventionally, determination of the toxicity of TTX among puffer has been using mouse as the animal of choice. However, this biological method is not suitable to obtain quantitative statements such as the toxin profile and the amount of single toxins (Diener et al. 2007). Therefore, chemical methods that have reliable and fast analysis are necessary. There are various methods available for testing of these substances. HPLC and LCMS are among the most powerful and sensitive tool for determination of TTX (Shoji et al. 2001; Yasumoto & Michishita 1985). More recent development is the application of LC-MS/MS in the detection and quantification of TTX and its analogues food matrices (Nakagawa et al. 2006).

Puffer fishes are very common in Malaysian waters, often caught in large numbers by trawlers or line fishing (Simon et al. 2009). There are at least 185 species of puffer fishes which distributed in 28 genera in the family Tetraodontidae (Oliveira et al. 2006). The most common species in Malaysia are *Lagocephalus lunaris, L. sceleratus* and *L. spadiceus,* consumed by some locals (Kan et al. 1987). In Sarawak, *Xenopterus naritus* or locally known as 'ikan buntal kuning' is considered a delicacy by the local community. Almost all puffer fish are poisonous and contain the poison (TTX) in their body parts. In Malaysia, although people do not eat puffer fish, many food poisoning cases due to ingestion of wild puffer fish have occurred. Most of these cases were caused by ingestion of contaminated puffer fish species. Since there is little published information on the toxicity of puffer fish species in Malaysia, this study was aimed to determine the concentration of TTX from different tissues of puffer fish. In this study, the toxicity of some fish specimens collected from Sabah and Sarawak waters was explored by the application of LC-MS/MS as an alternative method of the animal assays.

Materials and Methods

SAMPLE COLLECTION

A total of 154 puffer fishes consisted of 14 species from three different families was analysed in this study. They were two species from Diodontidae family (*Diodon holocanthus* and *Diodon hystrix*); one species from Ostraciidae family (*Ostracion nasus*) and 11 species from Tetraodontidae family (*Arothron immaculatus*, *Arothron manilensis*, *Arothron stellatus*, *Chelonodon patoca*, *Lagocephalus inermis*, *Lagocephalus lunaris*, *Lagocephalus sceleratus*, *Lagocephalus spadiceus*, *Tetraodon nigroviridis*, *Torquigener pallimaculatus* and *Xenopterus naritus*) collected from Pulau Mandi Darah, Sabah; Batang Sadong, Sampadi and Kuching Sarawak in October 2009. The samples were collected using a standard bottom trawl net or gill net. Immediately after collection, fish were kept in ice and stored at -20°C until delivered to Fisheries Research Institute, Penang for further analyses.

SAMPLE EXTRACTION AND PREPARATION

In the laboratory, the samples were thawed and sorted according to the species. The puffer fish identification was done based on the morphological characteristics (FRI 2004; Froese & Pauly 2011). After thawing, the fish specimens were weighed (33.2-2000 g) and measured for total length (10.0-60.0 cm) individually. Each specimen was dissected to remove the liver, skin and muscle. Toxin was extracted from each fish tissue according to Helbig and Luckas (2010) with slight modifications. Each tissue was minced and a small portion (1 g) was extracted with 3 mL of 0.03 M acetic acid using an ultrasonic probe (OMNI-Ruptor 4000, Georgia, USA) for 1 min. The homogenate was centrifuged at 5000 rpm for 15 min (Eppendorf 5430, Hamburg, Germany) and subsequently the supernatant was collected and transferred to a volumetric flask of 10 mL. The extraction step is repeated and the homogenate was centrifuged at 14000 rpm for 5 min (Eppendorf 5810 R, Hamburg, Germany). After centrifugation, the supernatant was transferred to a volumetric flask and made up with 0.03 M acetic acid. The sample extract was filtered through a 0.45 µm nylon membrane filter and the filtrate was analysed by an LC-MS/MS.

ANALYSES BY LC-MS/MS

Mass spectrometry was performed using a TSQ Quantum Discovery MAX model from Thermo Electron, USA consisting of an MS Surveyor pump with autosampler coupled to a Mass Spectrometer equipped with an electro spray ionisation (ESI) probe. Prior to analyses, mass calibration was done using 1,3,5 polityrosine in both negative and positive mode. Compound optimisation was carried out in the positive mode for the detection of the analyte based on its ionisation using a 1 ppm tuning standard solution of TTX. Optimal ion source and interface conditions were achieved at a spray voltage of 3800 V, sheath gas flow of 10 units, auxillary gas flow of 3 units, collision energy (CE) of 18, collision gas pressure of 1.5 mTorr and capillary temperature of 300°C. In the positive ionization, the ion transitions from the typical $[M+H]^+$ molecular ion of TTX (m/z 320) to the product ion (m/z 162) (quantifier) (Jang et al. 2010; Jen et al. 2008; Shoji et al. 2001; Yotsu-Yamashita et al. 2011) which was detected in selected reaction monitoring (SRM) mode with argon as the collision gas. Peak detection, data acquisition and the calibration graph plot were performed using the Xcalibur 2.1.0 software. The mass chromatogram was scanned at m/z 320 and TTX ($C_{11}H_{17}N_3O_8$, 319.27 Da) came out at about 7 min after sample loading (Figure 1).

TTX was separated on a 5 µm, 150×2.1 mm inner diameter ZIC-HILIC column (SeQuant, Haltern, Germany) with a guard column 5 µm, 20×2.1 mm (SeQuant, Haltern, Germany) at a flow rate of 250 µL/min. LC was performed with mobile phase A consisting of 10 mM ammonium formate and 10 mM formic acid in water. Mobile phase B contained 5 mM ammonium formate and 2 mM formic acid in acetonitrile/water (80/20, v/v). The gradient programme

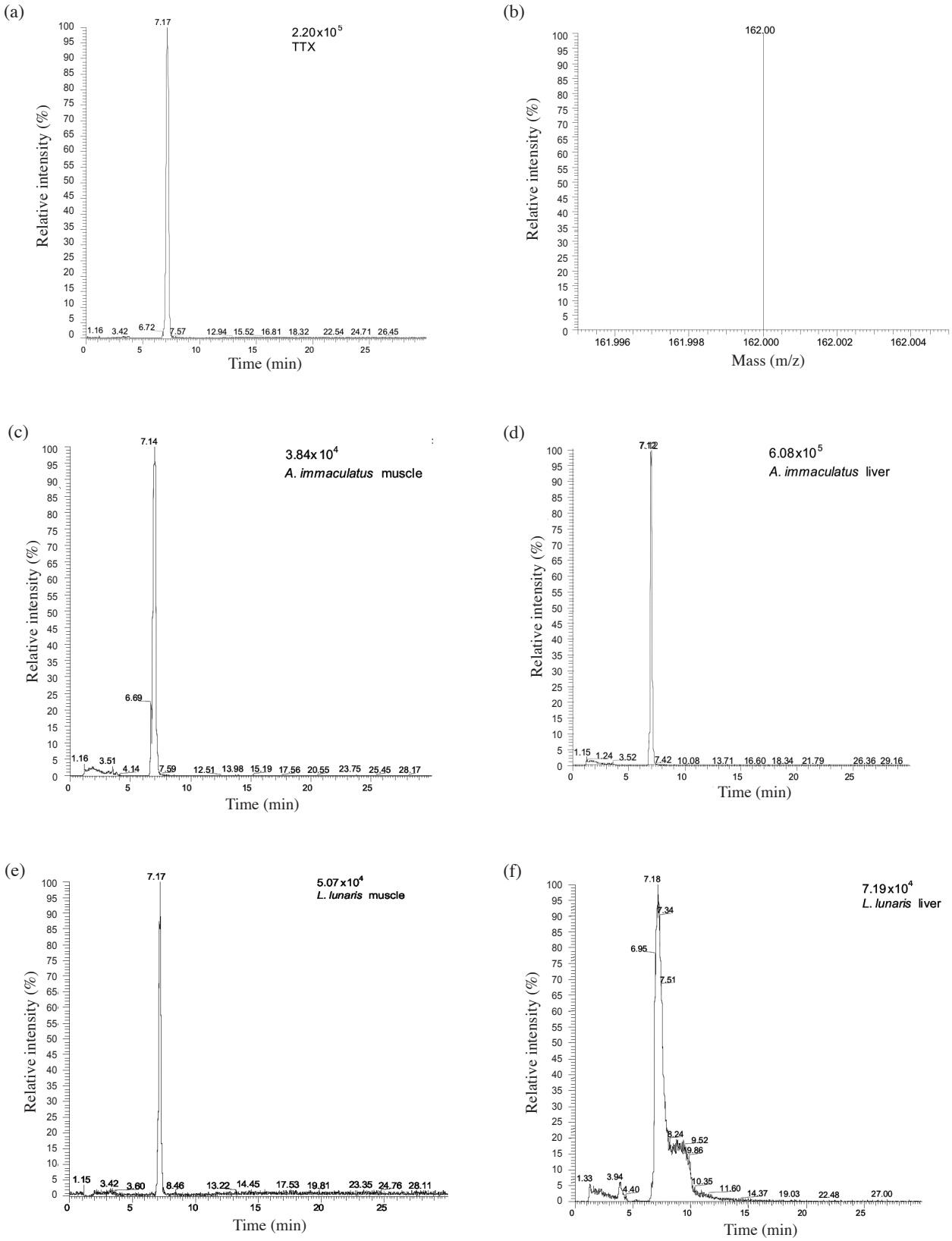


FIGURE 1. Full scan total ion current (TIC) chromatography for ion spray LC-MS/MS analysis of (a) standard of tetrodotoxin (TTX) (5 ng), (b) mass spectrum of daughter ion of TTX (c) extract of the muscle and liver of A. immaculatus (10 µL) and (d) extract of the muscle and liver of L. lunaris (10 µL)

was applied as described by Diener et al. (2007). The gradient elution started with 100% B and decreased to 65% B within 0.1 min, held over 7 min, then increased back to 100% B within 3 min and stayed stable for 15 min.

TOXIN STANDARDS

The TTX standard (Batch number APN09032-1-1) was purchased from Groupe Biomedix, Malaysia. Stock solution of TTX was prepared in 0.03 M acetic acid and stored at -20°C. Standard solutions of TTX (5, 10, 50, 100, 500 and 1000 ng/mL) were prepared by dilution of the stock solutions with 0.03 M acetic acid. Reference material for 4-epiTTX and 4,9-anhydroTTX were not available, therefore, the concentration for the analogs was calculated with the calibration equation for TTX. For monitoring TTX at m/z 320 Da in the total ion current (TIC) mode, the calibration curve was obtained using the standard TTX linear within the range of 5-1000 ng/mL (y = - 12635.4 + 6251.32x, r^2 = 0.9960) (Figure 2).

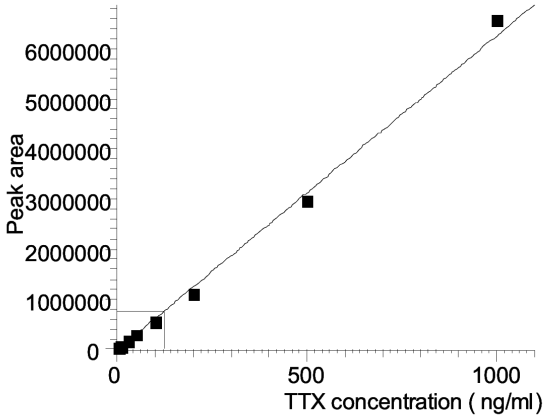


FIGURE 2. Calibration curves of TTX obtained by LC-MS/MS

STATISTICAL ANALYSIS

Data were analyzed by the statistical software of SPSS (Statistical Package for the Social Sciences) version 16.0 for Windows. One way analysis of variance (ANOVA) test was used to compare differences in the means of TTX of different species and tissues of puffer fish and followed by Duncan multiple range test analysis to determine the difference between species. Means ± SD were reported and considered different when $p<0.05$.

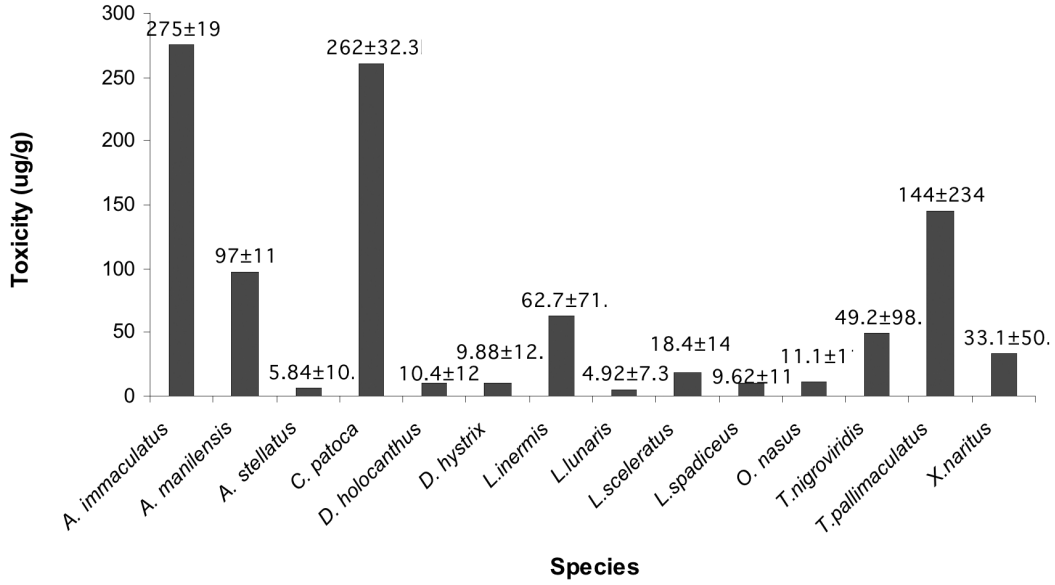
RESULTS AND DISCUSSION

Figure 1 shows the example of selected ion mass chromatogram of the liver and muscle extracts of *L. lunaris* and *A. immaculatus*. The extracts gave a peak at a retention time at almost consistent with that the standard TTX (7.17 min) (Figure 1(a)). This toxin was identified as TTX, which was confirmed by comparing with the TTX standard. TTX was detected in most all the species and tissues of the puffer fish. The mass spectrum of TTX with the formation of the daughter ion 162 is shown in Figure 1(b). The product ion m/z 162 was monitored because it had the most abundant and stable ion. Extracts of muscle and liver of *A. immaculatus* also contained TTX showed the same toxin profile as that of *L. Lunaris*'s extracts (Figure 1(c) & 1(d)).

The results of TTX concentrations in the different species of puffer fish are summarized in Figure 3. All of the species were found to contain TTX. There was significant differences in TTX concentrations for all puffer fishes ($p<0.05$). The highest mean value of TTX concentration was detected in *A. immaculatus* (275 μg/g) (Figures 3 & 5) and the lowest was detected in *L. lunaris* (4.92 μg/g) (Figures 3 & 6). The maximum amount of TTX in *D. holocanthus* and *D. hystrix* from the family of Diodontidae was 10.4 and 9.88 μg/g, respectively. Meanwhile the maximum amount of TTX in *O. nasus* from the family of Ostraciidae was 11.1 μg/g. From the ANOVA, the puffer fishes can be divided into three groups according to their TTX levels. There was significant difference in TTX concentration for *A. immaculatus* (275 μg/g) among the puffer fishes ($p<0.05$) but not significantly different from *C. patoca* (262 μg/g) (Figure 3). Among the *Lagocephalus* sp., *L. inermis* showed the highest mean TTX concentration (62.7 μg/g), followed by *L. sceleratus*, *L. spadiceus* and *L. lunaris* (18.4, 9.62 and 4.92 μg/g), respectively. However, there is no significant difference among them ($p>0.05$).

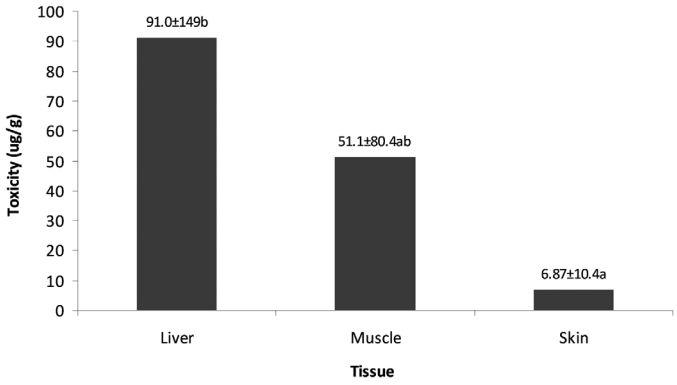
TTX concentrations obtained for each detected toxin in all tissue extracts of the whole puffer fish are shown in Figure 4. The TTX concentration in different tissues of puffer fishes showed that all the tissues were toxic and significantly different ($p<0.05$). In general, liver showed the highest TTX concentration among the tissues of puffer fishes (Figure 4). However, the TTX concentration is not significantly different from the muscle ($p>0.05$). Comparing the maximum TTX concentration found in the different tissues, the liver showed the highest at 91.0 μg/g followed by the muscle (51.1 μg/g) and the skin (6.87 μg/g).

Among the puffer fishes, *A. immaculatus* showed significantly highest toxic potency of 444 μg/g in its liver followed by *T. pallimaculatus* (260 μg/g) and *C. patoca* (246 μg/g) ($p<0.05$) (Table 1). *L. lunaris* (6.64 μg/g) showed the lowest TTX concentration in its liver. The lowest TTX concentration was detected in skin for all puffer fishes. Among the puffer fishes, TTX was not detected in the skin of *D. holocanthus*. TTX concentration in other puffer fish was below 10 μg/g except for *T. pallimaculatus* (29.1 μg/g) and *A. manilensis* (10.8 μg/g). Most of the muscle from the puffer fishes showed high concentration of TTX and significantly different with the highest was detected in *C. patoca* (277 μg/g) followed by *A. manilensis* (133 μg/g) and *A. immaculatus* (107 μg/g) ($p<0.05$). TTX was not detected in the muscle of *A. stellatus*, *D. hystrix* and *T.*



Results are Means ± standard deviation of duplicates.
Means with the same letter in each row are not significantly different (p>0.05)

FIGURE 3. Mean TTX concentration (µg/g) in different species of puffer fish



Results are Means ± standard deviation of duplicates.
Means with the same letter in each row are not significantly different (p>0.05)

FIGURE 4. Mean TTX concentration (µg/g) in different tissues from whole puffer fish

pallimaculatus. Comparison of the *Lagocephalus* sp., the TTX concentration of *L. lunaris* in the muscle was lower than the liver compared to the other *Lagocephalus* sp. (Table 1).

Six point calibration curves from mass spectrometric detection were calculated for TTX (Figure 2). Low LOD for TTX and a good correlation of the data for the concentration ranges tested could be observed in Table 2. Therefore, all TTX toxins present in independent of the puffer fish could be determined. Liquid chromatography results from the puffer tissue extracts and reference standard TTX gave the same fragmentation products after MS/MS showed that the puffer tissue extracts also contained TTX. TTX was a major toxic principle and showed the same toxin profile in the muscle and liver extract of *L. lunaris* by LC/ESI-MS (Nagashima et al. 2011). The retention time of puffer fish TTX sometimes was not identical to standard TTX. According to Nakamura and Yasumoto (1985), the difference was probably due to TTX exists as a mixture of its derivatives in puffer fish. Generally toxins in puffer fish are thought to be TTX, but paralytic shellfish poisoning toxins have also been detected in some puffer fish including freshwater from tropical regions (Kungsuwan et al. 1997; Ngy et al. 2009; Sato et al. 1997). Helbig and Luckas (2010) also reported the PSP toxins detected in *Takifugu poecilonotus* were composed of Neo, STX and dcSTX.

The present study showed that all of the puffer fish species collected from Sabah and Sarawak waters contained TTX, whereas toxin distribution between either the tissues of the same fish or other species was unequal. TTX levels in *L. spadiceus*, *T. nigroviridis* and *X. naritus* that was collected from Kuching was higher compared

TABLE 1. Average size and TTX concentration (μg/g) in different tissues from various species of puffer fishes

Family/Species	N	Total length (cm)	Total weight (g)	Toxicity (μg/g)		
				Liver	Muscle	Skin
DIODONTIDAE						
D. holocanthus	8	20.9±1.87	532±41.8	22.7±0.02[a]	21.1±0.57[ab]	nd
D. hystrix	4	46.3±5.17	904±40.2	20.8±0.0[a]	nd	-
OSTRACIIDAE						
O. nasus	4	27.1±1.65	174±44.5	10.9±13.7[a]	9.94±12.6[ab]	0.28±0.13[a]
TETRAODONTIDAE						
A. immaculatus	4	29.5±0.66	913±13.4	444±36.7[b]	107±12.2[ab]	-
A. manilensis	8	14.9±4.06	130±5.49	50.2±60.0[a]	133±155[b]	10.8±0.40[a]
A. stellatus	8	37.5±6.55	1350±554	11.2±14.1[a]	nd	0.44±0.12[a]
C. patoca	4	25.3±0.65	334±16.3	246±46.5[ab]	277±2.97[c]	-
T. pallimaculatus	8	14.8±0.75	80.5±5.69	260±303[ab]	nd	29.1±4.21[b]
L. inermis	8	23.6±1.92	261±31.6	46.0±55.7[a]	75.8±93.4[ab]	2.64±1.60[a]
L. sceleratus	12	49.3±5.22	445±30.0	24.7±2.33[a]	30.0±1.84[ab]	0.51±0.17[a]
L. lunaris	16	14.3±2.81	88.1±55.7	6.64±6.24[a]	4.96±9.76[ab]	3.15±7.45[a]
L. spadiceus	20	25.4±1.15	306±31.7	8.09±10.8[a]	8.71±12.1[ab]	1.71±6.71[a]
T. nigroviridis	22	13.9±2.32	126±73.3	49.8±134[a]	33.5±25.0[ab]	1.64±7.46[a]
X. naritus	28	19.6±4.26	206±133	45.2±68.8[a]	12.2±22.0[ab]	7.84±11.6[a]

Values are mean±s.d. of duplicates, nd = not detected, - = not tested
Means with the same letter in each row are not significantly different (p>0.05)

TABLE 2. Retention time, LOD (S/N 3:1) and equation of calibration curve for TTX analysed by LC-MS/MS

Toxin	m/z > m/z	Retention time (min)	Calibration equation	Correlation	LOD (ng on column)
TTX	320 > 162	7.17	Y = 6251.32x – 1263.4	0.9960	0.05

with the same species collected from Pulau Mandi Darah, Sabah, Sampadi and Batang Sadong, Sarawak (data not shown). Different toxin levels detected in the same species could be attributed to the fact that fish were caught in different areas. Rodriquez et al. (2012) demonstrated that toxin distribution within the tissues of six *L. sceleratus* specimens was different depending on fish size, area and season where fish were caught.

Among the 14 species examined, 11 species were belonging to the Tetraodontidae family, whereas only two and one species was belong to the Diodontidae and Ostraciidae family, respectively. From the study, it showed that most of the species of the family Tetraodontidae were toxic while all species of the family Diodontidae and Ostraciidae were weakly toxic. This result is in agreement with the study by Noguchi and Arakawa (2008) and Tani (1945). The distribution of TTX in puffer fish bodies appeared to be species-specific. In all puffer fish specimens from this study showed that TTX amount were found the highest in liver and muscle whereas the skin contained the lowest amount. The present study showed all of the species were found to be toxic for human consumption as the TTX level was more than 2 μg/g (10 MU/g) (Japan Food Hygiene Association 2005).

Generally, in marine species of puffer fish, liver and ovary showed the highest toxicity (more than 1000 MU/g), followed by intestines and skin (Noguchi et al. 2006) and the Japanese Ministry of Health, Labour and Welfare has prohibited these organs from being used for food from all species of puffer fish (Arakawa et al. 2010). However, muscles in many toxic species are regarded as edible (Mahmud et al. 2001). Even though *X. naritus* (33.1 μg/g), *T. nigroviridis* (49.2 μg/g) and *L. spadiceus* (9.62 μg/g) are harmless to humans and categorized as safe to eat (Che Nin et al. 2010; Froese & Pauly 2011), however in the present study, it could be considered unsafe for human consumption. Chulanetra et al. (2011) also demonstrated that *T. nigroviridis* from the Andaman seas were toxic and risky to consume. Excessive consumption (> 1 g) of these is harmful. The minimum lethal dose and minimum acute dose of TTX to human (wt. 50 kg) are estimated to be around 2 mg and 0.2 mg, respectively (Katikou et al. 2009). From this study, the muscles of *C. patoca* showed the highest toxicity followed by *A. manilensis*

FIGURE 5. *Arothron immaculatus*

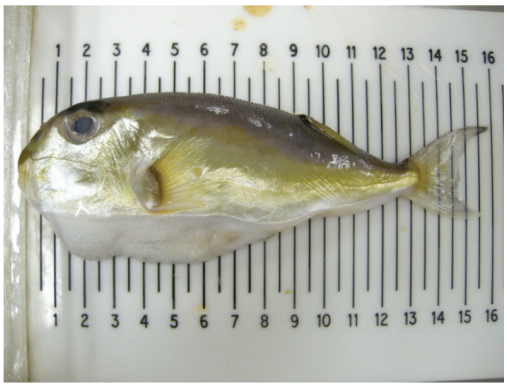


FIGURE 6. *Lagocephalus lunaris*

and *A. immaculatus*. Kungsuwan (1994) also found TTX in muscle, liver, skin and eggs of *A. immaculatus*, *C. patoca, L. lunaris, L. sceleratus* and *X. naritus* from the Andaman seas. However, TTX was not detected in *A. stellatus*, *D. hystrix*, *L. inermis* and *L. spadiceus* in his survey. In contrast, our study showed low level of TTX was detected in *A. stellatus*, *D. hystrix* and *L. spadiceus*. Most of the *Lagocephalus* species in the present study showed the highest toxicities in muscle, although the liver was frequently the most toxic tissues. Muscle also showed high toxicity levels in different *Lagocephalus* species in other studies (El-Sayed et al. 2003; Helbig & Luckas 2010; Noguchi et al. 2006). In contrast, *L. spadiceus* was found to be a non-toxic species (Berry & Hassan 1973; Brillantes et al. 2003; Che Nin et al. 2010; Kungsuwan 1994; Monaliza & Mohamad 2011) and this species was used as raw material to make fish balls by the local fish processing factories in Thailand (Brillantes et al. 2003). Among the puffer fishes in this study, *X. naritus* or locally known as 'ikan buntal kuning' is considered a delicacy by the local community in Sarawak. Although the muscle of *X. naritus* in this study was classified as weakly toxic (Noguchi et al. 2006), it is safe for consumption if prepared in a proper manner. In Betong, Sarawak, some of the local people had skills and experiences with the preparation of yellow puffer fish as they have eaten the fish for generations. However, there have been reports on the toxicity of *X. naritus* from Malaysia (Mohamad et al. 2008; Othman et al. 2006) and TTX was also found in all parts of tissue of *X. naritus* from the Andaman seas (Kungsuwan 1994).

Food poisoning was reported from different geographical regions due to puffer fish ingestion and the lethality was dependent on the concentration of TTX present in consumed fish tissues (Chou et al. 1994). Puffer fish poisoning is considered to be the common cause of fish poisoning along the coasts of Asia (Chew et al. 1983). To the best of our knowledge, this is the first report on the occurrence of TTX from Malaysian puffer fishes. In Malaysia, puffer fish is classified as trash fish which have no market value and they are not consumed by local people. However, some of the species have been considered edible and non-toxic. According to European Commission directive No 853/2004, fishery products derived from poisonous fish belonging to Tetraodontidae, Molidae, Diondontidae and Canthigasteridae family may not be placed on the market (EC 2004). Information on the safety of puffer fish consumption is insufficient. Therefore, their consumption or preparation as food to prevent poisoning required special regulations (Nunez-Vazquez et al. 2000). Preparing and cooking puffer fish require special technique about which most people are ignorant. During the preparation, the liver, gonads, intestines and skin which contain the highest level of toxin should be removed carefully and usually carried out by experienced individuals. Consumers of puffer fish should be educated on the potential risk of eating them, the warning symptoms and signs and when to seek medical advice.

CONCLUSION

In general, the present study provides general view on several common puffer species in Sabah and Sarawak waters, both on species distribution and toxicity as well as toxin component. Based on the findings, certain puffer species and the amount consumed may cause serious health effect on humans. However, for food safety purpose, it is necessary to have further study on seasonal which focus on individual variation of toxicity in each species. The results also indicated that LC-MS/MS assay is applicable for the determination of TTX and has the potential to replace the mouse bioassay.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. Mohammed Mohidin from the Fisheries Research Institute Sarawak Branch, Bintawa, Kuching, Sarawak and Ms. Ainah Puyong from Likas Fisheries Research Centre, Likas, Kota Kinabalu, Sabah for the supply of puffer fish samples. Appreciation goes to the Head of Fisheries Product and Food Safety Division, FRI, Penang, Hj. Rosly Hassan for his support and special thanks are extended to all technical staffs for their assistance in the study.

REFERENCES

Ahasan, H.A.M.N., Mamun, A.A., Karim, S.R., Bakar, M.A., Gazi, E.A. & Bala, C.S. 2004. Paralytic complications of puffer fish (Tetrodotoxin) poisoning. *Singapore Medical Journal* 45(2): 73-74.

Arakawa, O., Hwang, D.F., Taniyama, S. & Takatani, T. 2010. Toxins of pufferfish that cause human intoxications. In *Coastal Environmental and Ecosystem Issues of the East China Sea*, edited by Ishimatsu, A. & Lie, H.J. pp. 227-244.

Brillantes, S., Samosorn, W., Faknoi, S. & Oshima, Y. 2003. Toxicity of puffers landed and marketed in Thailand. *Fisheries Science* 69: 1224-1230.

Berry, P.Y. & Hassan, A.A. 1973. Comparative lethality of tissue extracts from the Malaysian puffer fishes *Lagocephalus lunaris lunaris*, *L. l. spadiceus* and *Arothron stellatus*. *Toxicon* 11: 249-254.

Che Nin, M., Norjuliana, M.N., Gan, L.H., Razak, L. & Samsur, M. 2010. Screening of tetrodotoxin in puffers using gas chromatography-mass spectrometry. *Journal of Chromatography A* 1217(47): 7455-7459.

Chew, S.K., Goh, C.H., Wang, K.W., Mah, P.K. & Tan, B.Y. 1983. Puffer fish (Tetrodotoxin) poisoning: Clinical report and role of anti-cholinesterase drugs in therapy. *Singapore Medical Journal* 24: 168-171.

Chou, S.S., Tsai, Y.H., Chang, P.C. & Hwang, D.F. 1994. Tetrodotoxin-associated food poisoning due to ingesting fish. *Journal Food and Drug Analysis* 2(1): 77.

Chua, H.H. & Chew, L.P. 2009. Puffer fish poisoning: A family affair. *Medical Journal of Malaysia* 64(2): 181-182.

Chulanetra, M., Soonkrung, N., Srimanote, P., Indrawattana, N., Thanongsaksrikul, J., Sakolvaree, Y., Chongsa-Nguan, M., Kurazono, H. & Chaicumpa, W. 2011. Toxic marine puffer fish in Thailand seas and tetrodotoxin they contained. *Toxins* 3: 1249-1262.

Diener, M., Christian, B., Ahmed, M.S. & Luckas, B. 2007. Determination of tetrodotoxin and its analogs in the puffer fish *Takifugu oblongus* from Bangladesh by hydrophilic interaction chromatography and mass-spectrometric detection. *Analytical Bioanalytical Chemistry* 389: 1997-2002.

EC (European Commission). 2004. Corrigendum to Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. *Official Journal of the European Union L* 139. pp L226/68 (25/6/2004).

El-Sayed, M., Yacout, G.A., El-Samra, M., Ali, A. & Kotb, S.M. 2003. Toxicity of the Red Sea pufferfish *Pleuranacanthus sceleratus* "El-Karad". *Ecotoxicology and Environmental Safety* 56(3): 367-372.

Fisheries Research Institute (FRI). 2004. *Field Guide to Selected Commercial Marine Fishes of Malaysian Waters*. Pulau Pinang, Department of Fisheries Malaysia.

Froese, R. & Pauly, D. 2011. FishBase. www.fishbase.org. Accessed on August 2011.

Helbig, T. & Luckas, B. 2010. Determination of tetrodotoxins (TTXs) and paralytic shellfish poisoning (PSP) toxins in puffer fish from Malaysia and Japan by application of a ZIC-HILIC column and mass spectrometric detection. *Current Development in Oceanography* 1(2): 69-83.

Isbister, G.K., Son, J., Wang, F., Maclean, C.J., Lin, C.S., Ujma, J., Balit, C.R., Smith, B., Milder, D.G. & Kiernan, M.C. 2002. Puffer fish poisoning: A potentially life-threatening condition. *Medical Journal of Australia* 177(11-12): 650-653.

Jang, J.H., Lee, J.S. & Yotsu-Yamashita, M. 2010. LC/MS analysis of Tetrodotoxin and its deoxy analogs in the marine puffer fish *Fugu niphobles* from the southern coast of Korea, and in the brackishwater puffer fishes *Tetraodon nigroviridis* and *Tetraodon biocellatus* from South East Asia. *Marine Drugs* 8: 1049-1058.

Japan Food Hygiene Association. 2005. Puffer toxin. In *Environmental Health Bureau, Ministry of Health and Welfare,* edited by Shokuhin Eisei Kensa Shishin. Manual for Methods for Food Sanitation Testing, Tokyo. pp. 661-673.

Jen, H.C., Lin, S.J., Tsai, Y.H., Chen, C.H., Lin, Z.C. & Hwang, D.F. 2008. Tetrodotoxin poisoning evidenced by solid-phase extraction combining with liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B* 871: 95-100.

Kan, S.K., Chan, M.K. & David, P. 1987. Nine fatal cases of puffer fish poisoning in Sabah, Malaysia. *Medicine Medical Journal of Malaysia* 42(3): 199-200.

Katikou, P., Georgantelis, D., Sinouris, N., Petsi, A. & Fotaras, T. 2009. First report on toxicity assessment of the Lessepsian migrant pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters (Aegean Sea, Greece). *Toxicon* 54: 50-55.

Kungsuwan, A. 1994. Survey on poisonous pufferfish in Andaman Seas. *Proceeding of the Seminar on Fisheries 1993*, Department of Fisheries, Bangkok, Thailand, 15-17 September 1993.

Kungsuwan, S., Arakawa, O., Promdet, M. & Onoue, Y. 1997. Occurrence of paralytic shellfish poisons in Thai freshwater puffers. *Toxicon* 35: 1341-1346.

Laobhripatr, S., Limpakarnjanarat, K., Sangwonloy, O., Sudhasaneya, S., Anuchatvorakul, B., Leelasitorn, S. & Saitanu, K. 1990. Food poisoning due to consumption of the freshwater puffer *Tetraodon fangi* in Thailand. *Toxicon* 28(11): 1372-1375.

Lin, S.J. & Hwang, D.F. 2001. Possible source of tetrodotoxin in the starfish *Astropecten scoparius*. *Toxicon* 39: 573-579.

Lyn, P.C.W. 1985. Puffer fish poisoning: Four case reports. *Medical Journal of Malaysia* 40(1): 31-34.

Mahmud, Y., Tanu, M.B., Takatani, T., Asayama, E., Arakawa, O. & Noguchi, T. 2001. *Chelonodon patoca*, a highly toxic marine puffer in Japan. *Journal of Natural Toxins* 10: 69-74.

Matsui, T., Taketsugu, S., Sato, H., Yamamori, K., Kodama, K., Ishi, A., Hirose, H. & Shimizu, C. 1990. Toxification of cultured puffer fish by the administration of tetrodotoxin producing bacteria. *Nippon Suisan Gakkaishi* 56(4): 705.

Miyazawa, K. & Noguchi, T. 2001. Distribution and origin of tetrodotoxin. *Journal of Toxicology Toxin Reviews* 20: 11-33.

Mohamad, S., Lim, P.T., Bojo, O., Nyanti, L., Ismail, N., Rahim, K.A. & M. Long, S. 2008. Toxicity of freshwater puffer fish *Xenopterus naritus* (Tetraodontidae) collected from East Malaysia. *IOC-WESTPAC Scientific Symposium*. 21st- 25th May 2008.

Monaliza, M.D. & Mohamad, S. 2011. Toxicity and toxin properties study of pufferfish collected from Sabah waters. *Health and the Environment Journal* 2(1): 14-17.

Mosher, H.S. & Fuhrman, F.A. 1984. Occurrence and origin of tetrodotoxin. In *Seafood Toxins*, edited by Edward, P.R. Washington, D.C.: American Chemical Society. pp. 333-344.

Nagashima, Y., Matsumoto, T., Kadoyama, K., Ishizaki, S. & Terayama, M. 2011. Toxicity and molecular identification of Green Toadfish *Lagocephalus lunaris* collected from Kyushu Coast, Japan. *Journal of Toxicology* 2011: 801285.

Nakagawa, T., Junho, J. & Yotsu-Yamashita, M. 2006. Hydrophilic interaction liquid chromatography-electrospray ionization mass spectrometry of tetrodotoxin and its analogs. *Analytical Biochemistry* 352: 142-144.

Nakamura, M. & Yasumoto, T. 1985. Tetrodotoxin derivatives in puffer fish. *Toxicon* 23: 271-276.

Ngy, L., Yu, C.F., Taniyama, S., Takatani, T. & Arakawa, O. 2009. Co-occurence of tetrodotoxin and saxitoxin in Cambodian marine pufferfish *Takifugu oblongus*. *African Journal of Marine Science* 31(3): 349-354.

Noguchi, T. & Arakawa, O. 2008. Tetrodotoxin – distribution and accumulation in aquatic organisms and cases of human intoxication. *Marine Drugs* 6: 220-242.

Noguchi, T., Arakawa, O. & Takatani, T. 2006. TTX accumulation in pufferfish. *Comparative Biochemistry and Physiology, Part D* 1: 145-152.

Nunez-Vazquez, E.J., Yotsu-Yamashita, M., Sierra-Beltran, A.P., Yasumoto, T. & Ochoa, J.L. 2000. Toxicities and distribution of tetrodotoxin in the tissues of puffer fish found in the coast of the Baja California Peninsula, Mexico. *Toxicon* 38: 729-734.

Oliveira, J.S., Fernandes, S.C.R., Schwartz, C.A., Bloch Jr., C., Melo, J.A.T., Pires Jr., O.R. & Freitas, J.C. 2006. Toxicity and toxin identification in *Colomesus asellus*, an Amazonian (Brazil) freshwater puffer fish. *Toxicon* 48: 55-63.

Othman, B., Khairul Adha, A.R., Samsur, M., Shabdin, M.L., Lee, N., Norhadi, I. & Lim, P.T. 2006. Toxicity of the yellow puffer fish *Xenopterus naritus* from Sungai Saribas Sarawak. *Research Bulletin, FRST* 1 (June). pp 5-6.

Pires, O.R., Sebben, A., Schwartz, E.F., Largura, S.W.R., Bloch, C., Morales, R.A.V. & Schwartz, C.A. 2002. Occurrence of tetrodotoxin and its analogues in the Brazilian frog *Brachycephalus ephippium* (Anura: Brachycephalidae). *Toxicon* 40(6): 61-766.

Rodriquez, P., Alfonso, A., Otero, P., Katikou, P., Georgantelis, D. & Botana, L.M. 2012. Liquid chromatography-mass spectrometry method to detect Tetrodotoxin and its analogues in the puffer fish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters. *Food Chemistry* 132: 1103-1111.

Sato, S., Kodama, M., Ogata, T., Saitanu, K., Furuya, M., Hirayama, K. & Kakinuma, K. 1997. Saxitoxin as a toxic principle of a freshwater puffer, *Tetraodon Fangi*, in Thailand. *Toxicon* 35: 137-140.

Shoji, Y., Yotsu-Yamashita, M., Miyazawa, T. & Yasumoto, T. 2001. Electrospray ionization mass spectrometry of tetrodotoxin and its analogs: Liquid chromatography/ mass spectrometry, tandem mass spectrometry and liquid chromatography/tandem mass spectrometry. *Analytical Biochemistry* 290: 10-17.

Simon, K.D., Mazlan, A.G. & Usup, G. 2009. Toxicity of puffer fishes (*Lagocephalus wheeleri* Abe, Tabeta and Kitahama, 1984 and *Lagocephalus sceleratus* Gmelin, 1789) from the east coast waters of Peninsular Malaysia. *Journal of Biological Sciences* 9(5): 482-487.

Tani, I. 1945. *Studies on Japanese Pufferfish in Association with Poisoning due to Ingestion of Them*. Tokyo: Teikoku, Tosho. p. 103.

Tsai, Y.H., Chao, S.M., Lin, G.T., Noguchi, T. & Hwang, D.F. 2004. Tetrodotoxins of the starfish *Astropecten vappa* collected from western Taiwan. *Fisheries Science* 70: 930-932.

Yasumoto, T. & Michishita, T. 1985. Fluorometric determination of tetrodotoxin by high performance liquid chromatography. *Agricultural and Biological Chemistry* 49: 3077-3080.

Yasumoto, T., Yasumura, D., Yotsu, M., Michishita, T., Endo, A. & Kotaki, Y. 1986. Bacterial production of tetrodotoxin and anhydrotetrodotoxin. *Agricultural and Biological Chemistry* 50(3): 793-795.

Yotsu-Yamashita, M., Jang, J.H., Cho, Y. & Konoki, K. 2011. Optimization for simultaneous analysis of tetrodotoxin, 4-epitetrodotoxin, 4,9-anhydrotetrodotoxin and 5,6,11-trideoxytetrodotoxin by hydrophilic interaction liquid chromatography-tandem mass spectrometry. *Forensic Toxicology* 29: 61-64.

Yu, C.F. & Yu, P.H.F. 2002. Are puffer fish more toxic in their spawning seasons? *Marine Biology* 140: 1053-1057.

M. Samsur
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
94300 Kota Samarahan, Sarawak
Malaysia

A. Mohd Nor Azman* & M. Othman
Fisheries Research Institute
11960 Batu Maung, Penang
Malaysia

*Corresponding author; email: mohayo01@yahoo.com

Received: 10 October 2012
Accepted: 16 September 2013