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RESEARCH ARTICLE

Tetrodotoxin and fatty acids contents of *Lagocephalus sceleratus* (Gmelin, 1789) collected in Antalya, Turkey, by MS/MS and GC/MS analyses

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Abstract

Tetrodotoxin (TTX) and fatty acids contents of five specimens of *Lagocephalus sceleratus* collected from Antalya on the Turkish Mediterranean coast were studied. TTX was determined by LC-MS/MS analysis in intestines, liver, ovary and muscle. Fatty acids were determined by GC/MS analyses. The oil content was not high (6%) but polyunsaturated n-3 and n-6 ratios were high.

Keywords: Tetradotoxin, TTX, fatty acids, *Lagocephalus sceleratus*, LC-MS/MS, GC/MS.

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Introduction

Tetrodotoxin (TTX) has been found in various marine organisms (Miyazawa and Noguchi 2001; Wu *et al.* 2005; Hwang *et al.* 2007) and also Shewanella red alga (Simidu *et al.* 1990). It was first isolated by Yokoo (1950) and its structure was elucidated by various authors (Yokoo 1950; Tsuda *et al.* 1964; Woodward 1964; Goto *et al.* 1965). It is a heterocyclic guanidine compound, chemically (4*R*,4a*R*,5*R*,6*S*,7*S*,8*S*,8a*R*,10*S*,12*S*)-2-azaniumylidene-4,6,8,12-tetrahydroxy-6-

(hydroxymethyl)-2,3,4,4a,5,6,7,8-octahydro-1*H*-8a,10-methano-5,7-(epoxymethanooxy) quin-azolin-10-olate. There are a number of natural analogues of TTX including, 4-epiTTX; 6-epiTTX; 11-deoxyTTX; 6,11-dideoxyTTX; 8,11-dideoxyTTX; 11-oxo-TTX; 11-norTTX-6,6-diol; 11-norTTX-6(*R*)-ol; 11-norTTX-6(*S*)-ol; chiriquitox; TTX-8-O-hemisuccinate; TTX-11-carboxylic acid (Bane *et al.* 2014).

Since the opening of the Suez Canal in 1869 more than 300 aquatic species have migrated from the Red Sea to the Mediterranean Sea (Bentur *et al.* 2008). Golani listed the migrating fish to the Mediterranean Sea including *Lagocephalus spadiceus*, *L. suenzensis*. *L. sceleratus* in (Antalya) Mediterranean Sea was recorded by Akyol *et al.* (Golani 2002; Akyol *et al.* 2005). TTX is a toxin found in various organs, such as muscle, intestine, liver testis, ovary and skin, of pufferfish *L. sceleratus* (Aydın 2011). The origin of TTX in fish is unknown. In contrary to wild fish, it was not found in cultured fish (Kono *et al.* 2008). The mortality rate is very high, thus it is forbidden to catch and sell this fish, although *L. sceleratus* has been consumed as food in Eastern Asia as well as in the Mediterranean countries (Nader *et al.* 2012; Rodriguez *et al.* 2012). TTX producing 36 bacterial strains has been isolated from the pufferfish (Yu *et al.* 2011).

Various methods have been published on the determination of TTX, among which LC-MS/MS technique most sensitive (Chen and Chou 1998; Jen *et al.* 2008; Jang *et al.* 2010; Chulanetra *et al.* 2011; Lin and Hwang 2012; Rodriguez *et al.* 2012; Silva *et al.* 2012). Biological mouse assay has also been used although not sensitive it is used as an assay for many marine toxic compounds (Brillantes *et al.* 2003; Wu *et al.* 2005; Sabrah *et al.* 2006; Noguchi and Arakawa 2008; Saoudi *et al.* 2008; Simon *et al.* 2009; Köşker *et al.* 2015).

The content of TTX analogues and isomers of *Lagocephalus* sp. and *L. sceleratus* from the Mediterranean Sea were determined by LC-MS/MS (Rodriguez *et al.* 2012; Kızılkaya 2014).

Many papers were published on fatty acids contents of fish oils which are important for human health. Especially n-3 fatty acids reduce the risk of heart attacks (Daviglus *et al.* 1997). Polyunsaturated fatty acids (PUFA) Eicosapentaenoic acid (EPA) 20:5 n-3 and docosohexaenoic acid (DHA) 22:6 n-3: important fatty acids of *L. sceleratus* were investigated in the Mediterranean Sea samples (Özoğul *et al.* 2009; Nurullahoğlu and Ulusoy 2011; Köşker 2014) and 26 species of pufferfish including *Lagosephalus* spp. (*L. inermis*, *L. wheeleri*, *L. glovevi*, *L. guntheri*, *L. spadiceus* and *L. lunaris*) imported from Korea, China and Bangladesh were investigated (Oyaizu *et al.* 2000).

In this paper Tetradotoxin and fatty acids contents of *Lagocephalus sceleratus* var. in Turkish coast of Mediterranean Sea are reported.

Materials and Methods

Five samples of *L. sceleratus* were caught by gillnets and trammel nets in the Gulf of Antalya in February 2014. All captured fishes were transferred to the laboratory maintaining cold chain.

Extraction of TTX from organs and LC-MS/MS conditions for TTX determination
Following the method by Jang *et al.* (2010), 2.6 g ovary, 10 g intestine, 35 g muscle, 1 g liver tissues were extracted. Each sample was homogenized with 0.05 M acetic acid contained methanol in homogenizer and then heated 5 min in water bath, centrifuged at 4000 rpm for 15 min and supernatant phase was separated and filtered. 2 mL aliquot was applied on the reverse phase column C18 (purchased from Macherey-Nagel) which was equilibrated with water after methanol. The first passing solution was discarded and following solution was collected with chloroform. Aqueous phase was separated and applied for LC-MS/MS analysis.

Spectroscopic analysis of aqueous part was performed Applied Biosystems 3200 Q-Trap LC-MS/MS instrument equipped with an ESI ion source with direct-injection to mass spectrometry at positive ionization mode. Enhanced Product Ionization Mode (EPI) chosen for TTX analysis which enabled to select and break a specific molecular weight.

Extraction of fatty acids from flesh
After skin and internal organs were separated, muscle fillets were cut into small pieces using a knife then homogenized using a homogenizer. Homogenized clean spineless fillets (100 g) were extracted with n-hexane using a Soxhlet apparatus for 6 h. The lipid content was determined after removal of solvent and kept at -18 ^{o}C. Methyl esters of fatty acids were prepared through modified method of Joseph and Achman (1992) and Aslan *et al.* (2009), and analyzed by GC-MS (Agilent 5975 GC-MSD). Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) was used with helium as carrier gas (0.8 mL min^{-1}). GC oven temperature was kept at 60 $^{\circ}$C for 10 min and programmed to 220 $^{\circ}$C at a rate of 4°C min^{-1} and kept constant at 220 $^{\circ}$C for 10 min and then programmed to 240 $^{\circ}$C at a rate of 1 ^{o}C min^{-1}. Split ratio was adjusted 40:1. The injector temperature was at 250 $^{\circ}$C. Ionization energy was 70 eV. Mass range was from m/z 35 to 450. FID temperature was 300 $^{\circ}$C.

The amount of each fatty acid was estimated by comparison of their mass spectra with those in the Baser Library of Essential Oil Constituents, Adams Library (Adams 2007), MassFinder Library (Hochmuth 2008), Wiley GC/MS Library (McLafferty and Stauffer 1989) and confirmed by comparison of their retention indices. Alkanes were used as reference points in the calculation of relative

retention indices (RRI) (Curvers *et al.* 1985). Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

Results and Discussion

The mass spectra of *L. sceleratus* organs are shown in Figure 1. Various authors reported the similar results on these as 230, 304, 302, 290 (Shoji *et al.* 2001), 320, 302, 251 (Wu *et al.* 2005), 320, 302, 280, 260 (Jen *et al.* 2008), 320, 304, 302, 288 (Jang *et al.* 2010), 320, 302, 284, 256 (Rodriguez *et al.* 2012), 320, 302 (Silva *et al.* 2012), 320, 302 (Chulanetra *et al.* 2011).

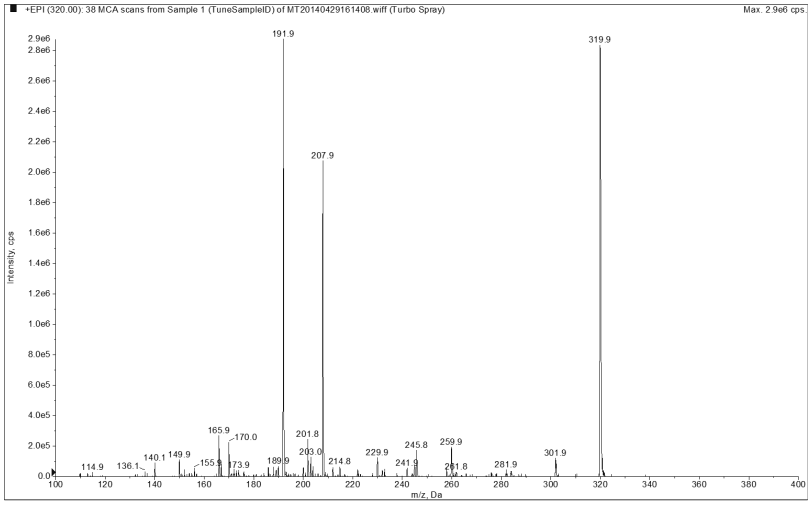

Figure 1a. MS spectrum of compound m/z 320 (M+H) extracted from muscle.

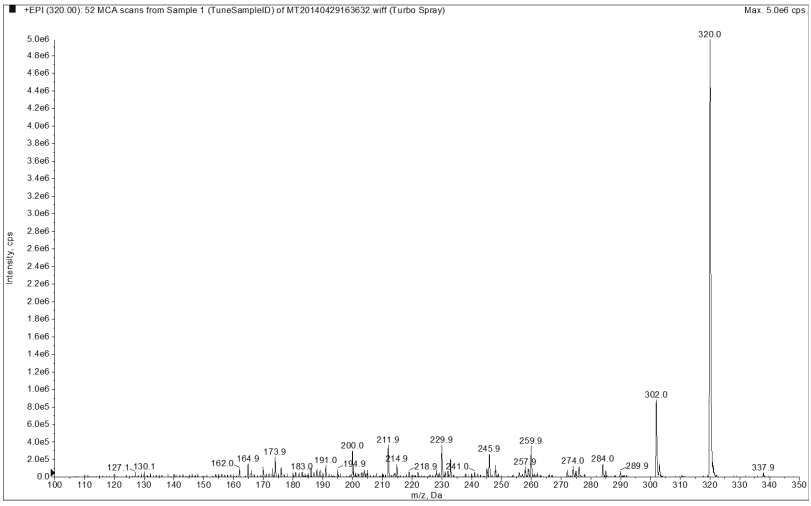

Figure 1b. MS spectrum of compound m/z 320 (M+H) extracted from intestine.

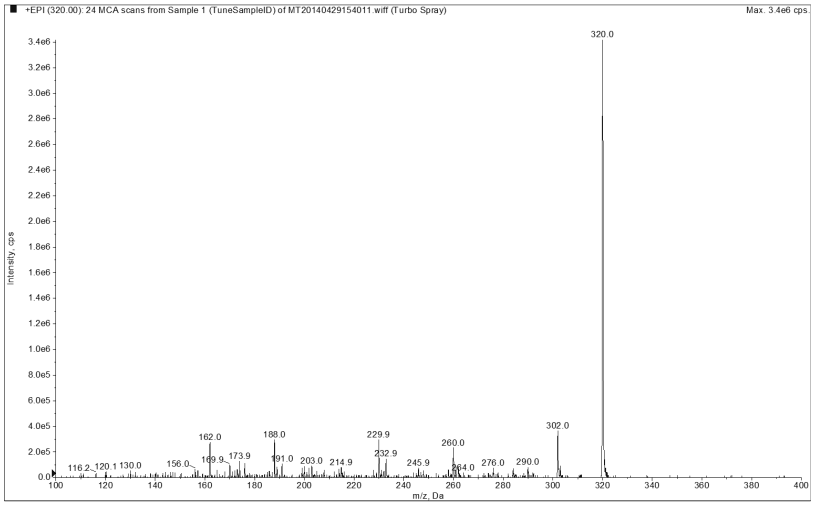

Figure 1c. MS spectrum of compound m/z 320 (M+H) extracted from liver.

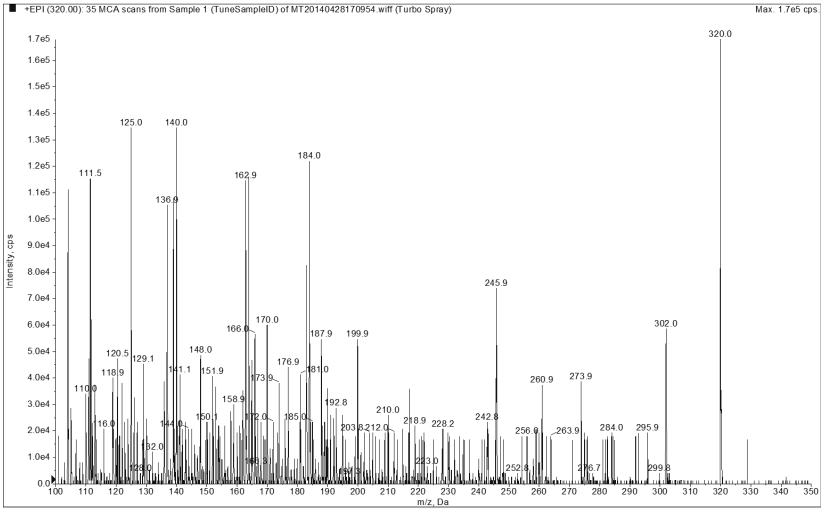

Figure 1d. MS spectrum of compound m/z 320 (M+H) extracted from ovary.

In summary, the dominant peaks are 320, 302, 290 and 260. The dominant peaks of tetradotoxin in examined tissues were found as 320, 302, 280 in muscles, 320, 302, 284, 2 in intestines, 320, 302, 280 in livers, 320, 302, 284, 200 in ovary. The comparison of LC-MS/MS results with the findings of indicated literature proved that muscle, liver, intestine and ovary contain TTX.

Table 1. Fatty acid compositions of flesh (g/100 g lipids)

Fatty acids	g/100 g lipids
C12:0	-
C13:0	-
C14:0	-
C15:0	NSA
C16:0	6.1
C17:0	-
C18:0	6.8
C19:0	-
C20:0	-
C21:0	-
C24:0	-
∑ SFA	12.9
C16:1 (*n*-7)	NSA
C17:1 (*n*-7)	-
C18:1 (*n*-9)	13.7
C18:1 (*n*-7)	3.2
C20:1 (*n*-9)	-
C22:1 (*n*-11)	NSA
C24:1(*n*-9)	-
∑ MUFA	16.9
C18:2 (*n*-6)	1.5
C18:3 (*n*-6)	-
C18:3 (*n*-3)	-
C18:4 (*n*-3)	-
C20:2 (*n*-6)	-
C20:4 (*n*-6)	6.7
C20:5 (*n*-3) (EPA)	NSA
C22:5 (*n*-3)	-
C22:6 (*n*-3) (DHA)	46.8
∑ PUFA	55.0
∑ Unsat.	71.9
Sat./Unsat.	0.1
∑ MUFA/∑ PUFA	0.31
∑ *n*-3 PUFA	46.8
∑ *n*-6 PUFA	4.7
n-3/*n*-6	0.9
DHA/EPA	-
Lipid *	0.6
Unidentified	15.2

-: not detected; NSA: no significant amount (<0.01 g/100 g lipids), * (g/100 g sample)

Fatty acid compositions are shown in Table 1. The lipid level of this species is relatively very low compared to other fish (Love 1970) as 0.6% in our findings, comparable to the other studies reported between 0.2-1.8% (Özoğul *et al.* 2009; Nurullahoğlu and Ulusoy 2011; Aydın *et al.* 2013; Köşker 2014). The recent studies and our result indicate the composition of fatty acids in *L. sceleratus*

collected from Turkish coast of Mediterranean Sea are mainly C16, C18, C18:1 (n-9), C18:1 (n-7), C18:2 (n-6), C20:4 (n-6), C20:5 (n-3) (EPA), C22:5 (n-3), C22:6 (n-3) (DHA), however, the amounts temporally vary as indicated in two reports (Nurullahoğlu and Ulusoy 2011; Aydın *et al.* 2013). The total saturated fatty acid (SFA), mono unsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were determined as 12.9, 16.9, 55.0%, respectively. Total percentage of omega-3 PUFAs was found as (only DHA was found) 46.8%. Notably C20:5 (n-3) (EPA) was not found significant amount for the recent study unlike to the previous reports. These ratios were comparable with other studies (Özoğul *et al.* 2009; Nurullahoğlu and Ulusoy 2011; Aydın *et al.* 2013; Köşker 2014).

Conclusion

TTX was detected in edible muscle, liver, intestine and ovary of *L. sceleratus*. Oil and its fatty acids were determined. It was found that the total lipid content of *L. sceleratus* was low as indicated previously.

Antalya, Türkiye kıyılarından yakalanan *Lagocephalus sceleratus* (Gmelin, 1789)'in MS/MS ve GC/MS analizleriyle tetrodotoksin ve yağ asitleri içeriği

Öz

Türkiye kıyılarından Antalya'da yakalanan beş *Lagocephalus sceleratus* (Gmelin, 1789)'in MS/MS ve GC/MS analizleriyle tetrodotoksin ve yağ asitleri içeriği çalışıldı. LC-MS/MS analizi ile barsak, karaciğer, ovar ve kasta TTX bulundu. GC/MS analizleriyle yağ asitleri tespit edildi. Yağ içeriği yüksek değil fakat çoklu doymamış n-3 and n-6 oranları yüksektir.

Anahtar Kelimeler: TTX, tetrodoksin, yağ asitleri, *Lagocephalus sceleratus*, LC-MS/MS, GC/MS.

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