Advanced Underwater Sciences
http://publish.mersin.edu.tr/index.php/aus/index
e-ISSN: 2791-8734

Tetrodotoxin (TTX) extraction methods applied for the Silver Cheeked Toadfish (*Lagocephalus sceleratus* (Gmelin, 1789))

Hasan Deniz Akbora [*1], **Deniz ayas** [2]

[1] *Eastern Mediterranean University, Faculty of Arts and Sciences, Famagusta, North Cyprus*
[2] *Mersin University, Faculty of Fisheries, Department of Processing Technology, Mersin, Turkey*

Keywords
Pufferfish,
Neurotoxin,
Aquatoxicology,
Technique,
Reliable.

ABSTRACT

Silver cheeked toadfish is one of the most popular invasive species that originated from the Red Sea. They can inflate themselves inside or outside the sea with water, making them hard to swallow by predators. This fish also has a neurotoxin named Tetrodotoxin in various body tissues and makes them non-edible for human consumption. There are many death reports in the Red Sea and the Mediterranean Sea because of the consumption of this fish by humans. In this review, we have summarized the techniques used to detect the amount of Tetrodotoxin in the Silver Cheeked Toadfish. The main aim of this study is to give researchers a fast source of TTX extraction procedures.

1. INTRODUCTION

Lagocephalus sceleratus which is also known as the Silver Cheeked Toadfish is a species of fish belonging to the family Tetraodontidae. This bony fish species are originated from Indo-Pacific and can reach up to a total length of 110 cm (Boustany et al. 2015; Yaglioglu et al. 2011; Akbora et al. 2020). In case of danger, they collect air or water in their bodies, grow in volume, and avoid being swallowed by predators (Golani et al., 2006).

What is Tetrodotoxin (TTX)?

It is a non-protein neurotoxic molecule (see fig. 1) found in some terrestrial and marine animals. It prevents nerve impulses by blocking voltage-gated sodium channels (Nzoughet et al. 2013).

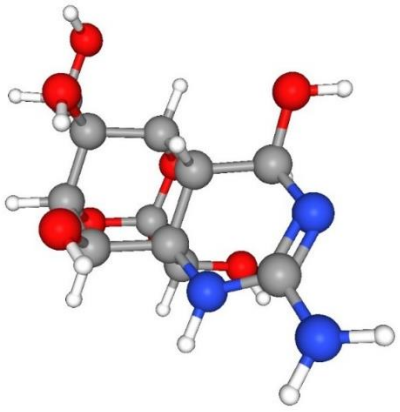

Figure 1. 3D structure of Tetrodotoxin molecule (https://pubchem.ncbi.nlm.nih.gov/compound/6324668#section=3D-Conformer).

* **Corresponding Author**
*(hdakbora@hotmail.com) ORCID ID 0000-0002-6647-866X
(ayasdeniz@mersin.edu.tr) ORCID ID 0000-0001-6762-6284

Cite this article;
Akbora, H. D. & Ayas, D. (2022). Tetrodotoxin (TTX) extraction methods applied for the Silver Cheeked Toadfish (Lagocephalus sceleratus (Gmelin, 1789)). *Advanced Underwater Sciences*, 2(1), 18-22

Review Article **Received: 14/09/2021; Accepted: 21/03/2022**

Some terrestrial animals like Colostethus inguinalis (Cope, 1868) (Common Rocket Frog), has TTX on their body (Daly et al., 1994). In addition, most of the fish species belonging to the Tetraodontidae family can harbor TTX in their body. Another popular marine animal having TTX in its body is Greater Blue-Ringed Octopus Hapalochlaena lunulata (Quoy & Gaimard, 1832). They can transfer TTX from their salivary glands. There are some human toxification issues reported from Japan (Asakawa et al., 2019).

Analysis Techniques

- Extraction: According to Evans (1969), the tetrodotoxin molecule is quite soluble in dilute acetic acid; besides, It is soluble in small amounts in water, ether, and ethanol. In addition, when exposed to strong acids or bases, its molecular structure deteriorates. For these reasons, diluted acetic acid is used for extraction in all methods, regardless of the analysis method. After extraction, to stabilize the molecule or, in methods needs to use enzymes, pH adjustment can also be made by researchers.

- Biological Analysis Methods: ELISA (Enzyme-Linked Immunosorbent Assay) and MBA (mouse bioassay) techniques are biological techniques commonly used in tetrodotoxin analysis. Since enzymes are used in the ELISA technique and mice are used in the MBA technique, these techniques are classified as biological.

- Chemical Analysis Methods: IR (infrared spectroscopy), NMR (nuclear magnetic resonance), GC-MS (Gas Chromatography-Mass Spectrometry), LC-FLD (Liquid chromatography fluorescence detector) and LC-MS (Liquid Chromatography-Mass Spectrometry) techniques are reliable for tetrodotoxin analysis, and used by many researchers (Bane et al.2014).

Techniques used for tetrodotoxin (TTX) analysis in *L. sceleratus*

Although there are many techniques that can be used for tetrodotoxin analysis, for *L. sceleratus*; MBA (Kosker et al.2016; Katikou et al. 2009), ELISA (Akbora et al. 2020), GC-MS (Man et al.2010) and LC-MS (Rodriguez et al. 2012; Azman et al. 2014; Kosker et al. 2016) techniques were used.

- MBA- Mouse Bioassay: Kosker et al. (2016) analyzed the amount of TTX using the MBA method and LC-MS / MS methods in their study on *L. sceleratus*. If the procedure is explained with the help of the steps in this study;

Extraction: 10 grams of tissues to be analyzed are weighed and placed in 25 ml of 0.1% CH_3COOH solution and homogenized for 10 minutes at 2400 rpm. The mixture is kept in a 100ºC hot water bath for 10 minutes and kept until it comes back to room temperature. The cooled mixtures are filtered with the help of 110 mm filter paper and the residues remaining on the paper are washed with 0.1% acetic acid. All the filtered and washed solutions are combined and filled with 0.1% acetic acid to make up to 50 ml. Each 1 ml of the prepared solution corresponds to 0.2 g of tissue. Mice allowed to be used in biochemical experiments are listed in Labome (2019). Kosker et al. (2016) used the Swiss Webster Albino mouse in their research. From the mixture obtained, 1 ml per tissue is taken and injected into 3 different mice intraperitoneally. With the help of a stopwatch, the time between the time of injection and the time of death is recorded. TTX levels are calculated in units of MU (mouse unit) with the help of the table given in Kawabata, (1978).

- ELISA: Enzyme-Linked Immunosorbent Assay (ELISA) method is basically based on the immunological working principle based on the antibody-antigen relationship. It is a technique that can be used to determine the presence of antigen, antibody, protein, peptide, or hormone present in a sample. The most common modification of the ELISA technique, which has many different modifications, used in TTX analysis is competitive ELISA methods (Akbora 2018).

Extraction: TTX extraction is similar in all techniques due to the chemical properties of the molecule. Basically, when using the ELISA method, the extraction steps are as follows:

Disintegrating the tissue with the help of a homogenizer in 0.1% acetic acid and keeping the mixture in a boiling hot water bath. Making the pH adjustment determined in the ELISA Kit procedure. Centrifugation is followed by the removal of the supernatant.

Competitive ELISA modifications used in TTX analysis:

Indirect Competitive ELISA (ic ELISA)

In this modification of competitive ELISA, the TTX molecule in the sample is competing with a pre-coated antigen to bind with the primary antibody.

Basically, when you add your extracted TTX solution into a microtiter tube and add primary antibody; TTX molecules inside the sample solution and precoated TTX molecules inside the tube start to compete. If the TTX molecules inside the sample are too much, they will bind a lot of antibodies. If there is not too much TTX in the sample, precoated TTX molecules will attract primary antibodies. After the washing step, only antibodies bind to precoated TTX molecules will remain inside the tube. The next step is the addition of enzyme-linked secondary antibody which is also known as a detection antibody. Detection antibodies are specific for primary antibodies. So, during incubation detection antibodies binds to primary antibodies. After washing, only the detection

antibodies bind to primary antibodies will remain inside the tube. The last step is the addition of enzyme-substrate. There will be a reaction between enzyme and substrate and a color change observed. The color change is a measurable product to detect the amount of TTX inside the original sample (See fig.2).

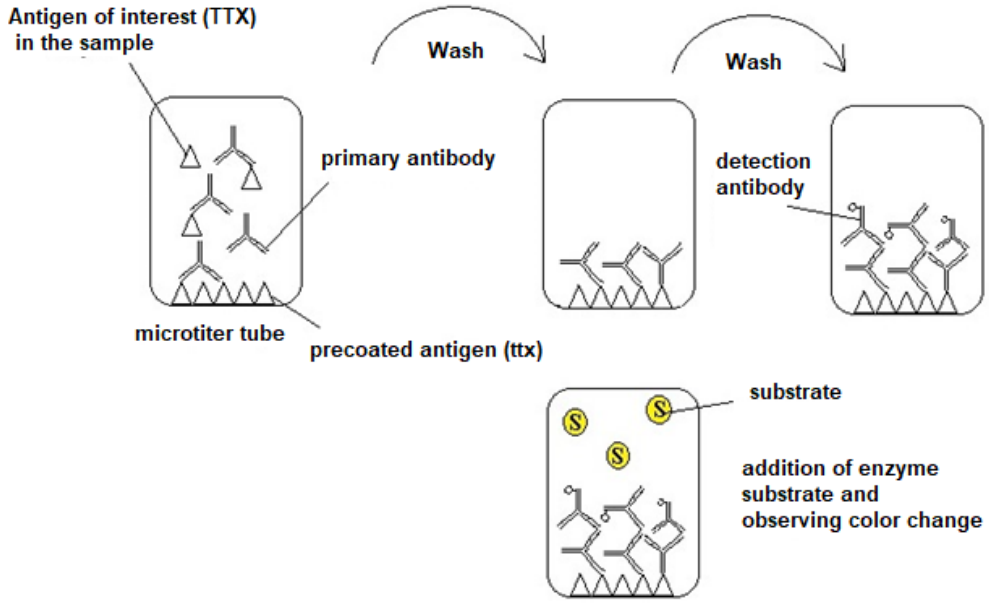

Figure 2. Schematisation of the Indirect Competitive ELISA method (This figure has been created according to Zhao et al. (2006)).

Direct Competitve ELISA (dc ELISA)

This modification of ELISA is quite similar with the ic ELISA. In this method, there is no primary antibody. TTX molecules inside the sample and precoated TTX molecules are competing to bind the detection antibody. After the washing step, and adding enzyme-substrate, there will be a color change which is the measurable product of the dc ELISA (See fig.3).

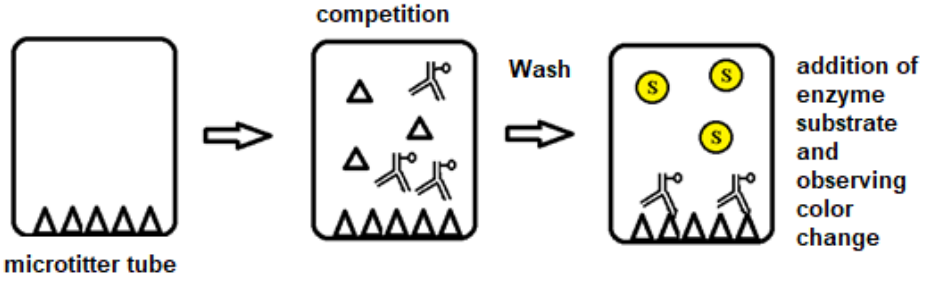

Figure 3. Schematisation of the Direct Competitive ELISA method (This figure has been created according to Zhao et al. (2006)).

In both competitive ELISA modifications, the color change and the amount of TTX inside the sample are inversely proportional. So, if the color change in a tube is higher than the other one, the amount of TTX inside the sample is less.

- GC-MS (Gas Chromatography-Mass Spectrometry): Because the TTX molecule is not volatile, this technique involves a different step compared to other techniques. This is the step of making the molecule volatile. In a study conducted in Malaysia, TTX amounts in muscle tissue were analyzed using the GC-MS method (Man et al., 2010). Again in Malaysia, another researcher analyzed TTX amounts with the LC-MS method and obtained results approximately 17.5 times higher than the GS-MS method (Azman et al., 2014). Bane et al. (2014) stated in their review that the GS-MS method is not a reliable technique in terms of TTX analysis and that it is a waste of time and money. For this reason, the details of the GC-MS technique were not discussed in our review, only it was mentioned that this technique has also been studied before.

- LC-MS (Liquid Chromatography-Mass Spectrometry): This technique has been accepted as the most widely used and most reliable method for researching TTX and its derivatives worldwide.

Extraction: As with all other techniques, the first few steps of TTX extraction from tissues are similar. Tissues

are homogenized in 0.1% acetic acid solution. Homogenized tissues are mixed with the help of a vortex and an ultrasonic mixer. These steps are repeated 2 times, and the final products are combined and centrifuged. The supernatants obtained are rounded to the desired volume by adding acetic acid. 1 ml of the final mixture is taken and cleaned using a solid-phase extraction cartridge (SPE), which was previously filled with methanol and distilled water. The cleaned sample is diluted by adding 100% methanol until reaching the desired volume. Each sample is concentrated by heating until dry and resuspended in 1 ml of methanol. 100 µl are taken from the samples, passed through 0.45 µm filters, and made ready for analysis. Extracts are given one by one to the LC-MS system and analyzed according to their mass/charge ratio (Rodriguez et al. 2012; Azman et al. 2014; Kosker et al. 2016).

2. DISCUSSION

Many techniques are used worldwide for TTX analysis in L. sceleratus. Techniques may differ according to the technical equipment infrastructure and budget of the researchers. For example, an LC-MS/MS system can be installed with a budget of around $145000, and the ELISA system with a budget of $1000-10000 (depending on the brand and features). Researchers with limited budgets can use techniques suitable for their budgets. Considering the toxin's chemical structure, it has been observed that the gas chromatography method is not reliable.

Ethical approval is required for the technique with the mouse. The use of experimental animals is prohibited in some countries if analysis can be made with alternative methods.

Although the ELISA method can be used as an alternative technique for TTX analysis; problems that may occur during the blocking of ELISA cuvettes cause incorrect antibody binding, and misleading color changes can be seen. In addition, plates containing ELISA wells may lose their properties when they are kept for a long time and may cause faulty color change.

LC-MS / MS method is the most common and reliable method used for TTX analysis. The only disadvantage of the LC-MS / MS method is that it is quite expensive to set up. Both methods (ELISA and LC-MS) can give proper results under suitable conditions. In selecting the analysis method and equipment, it is important to choose the device that will work in the longest term by considering the future research fields. It is important to establish a laboratory infrastructure accordingly.

Author contributions

The authors contributed equally to the article.

Conflicts of interest

The authors declare that they have no conflict of interest.

Statement of Research and Publication Ethics

For this type of study formal consent is not required.

REFERENCES

Akbora, HD., Kunter, İ., Erçetin, T., Elagöz, AM. & Çiçek, BA. (2020). Determination of tetrodotoxin (TTX) levels in various tissues of the silver cheeked puffer fish (*Lagocephalus sceleratus* (Gmelin, 1789)) in Northern Cyprus Sea (Eastern Mediterranean). *Toxicon*, 175, 1-6.

Asakawa, M., Matsumoto, T., Umezaki, K., Kaneko, K., Yu, X., Gomez-Delan, G., Tomano, S., Noguchi, T. & Ohtsuka, S. (2019). Toxicity and Toxin Composition of the Greater Blue-Ringed Octopus Hapalochlaena lunulata from Ishigaki Island, Okinawa Prefecture, Japan. *Toxins*, 11(5), 245.

Azman, AMN., Samsur, M. & Othman, M. (2014). Distribution of tetrodotoxin among tissues of pufferfish from Sabah and Sarawak waters. *Sains Malaysiana*, 43(7), 1003-1011.

Bane, V., Lehane, M., Dikshit, M., O'Riordan, A. & Furey, A. (2014). Tetrodotoxin: Chemistry, toxicity, source, distribution and detection. *Toxins*, 6(2), 693-755.

Boustany, L., El Indary, S. & Nader, M. (2015). Biological characteristics of the Lessepsian pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) off Lebanon. *Cah. Biol. Mar*, 56, 137-142.

Daly, JW., Gusovsky, F., Myers, CW., Yotsu-Yamashita, M. & Yasumoto, T. (1994). First occurrence of tetrodotoxin in a dendrobatid frog (Colostethus inguinalis), with further reports for the bufonid genus Atelopus. *Toxicon*, 32(3), 279-285.

Evans, MH. (1969). Mechanism of saxitoxin and tetrodotoxin poisoning. British Medical Bulletin, 25(3), 263-267

Golani, D., Öztürk, B. & Başusta, N. (2006). Fishes of the Eastern Mediterranean–Turkish Marine Research Foundation (Publication No. 24). Istanbul, Turkey.

Akbora, HD. (2018) Kuzey Kıbrıs Denizlerinde yaşayan benekli balon balığı (*Lagocephalus sceleratus* (Gmelin 1789))'nın çeşitli dokularında bulunan tetrodotoksin (TTX) düzeylerinin belirlenmesi (Master Thesis, Ankara Üniversitesi, 16-19.

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(1), 50-55.

Kawabata, T. (1978). Tetrodotoxin. In Veterinary Sanitation Division, Environmental Health Bureau, Ministry of Health and Welfare (Ed.), Food hygiene examination manual (shokuhin eisei kensa shishin) (pp. 223–241). Tokyo: Japan Food Hygiene Association.

Kosker, AR., Özogul, F., Durmus, M., Ucar, Y., Ayas, D., Regenstein, JM. & Özogul, Y. (2016). Tetrodotoxin levels in pufferfish (*Lagocephalus sceleratus*) caught in the Northeastern Mediterranean Sea. *Food Chemistry*, 210, 332-337.

Labome. (2019) Laboratory Mice and Rats. [ONLINE] Available at: https://www.labome.com/method/Laboratory-Mice-and-Rats.html. [Accessed 27 May 2021].

Man, CN., Noor, NM., Harn, GL., Lajis, R. & Mohamad, S. (2010). Screening of tetrodotoxin in puffers using gas chromatography–mass spectrometry. *Journal of Chromatography A*, 1217(47), 7455-7459.

Nzoughet, JK., Campbell, K., Barnes, P., Cooper, KM., Chevallier, OP. & Elliott, CT. (2013). Comparison of sample preparation methods, validation of an UPLC–MS/MS procedure for the quantification of tetrodotoxin present in marine gastropods and analysis of pufferfish. *Food Chemistry*, 136(3-4), 1584-1589.

PubChem. (2019). Maculotoxin | C11H17N3O8 - PubChem. [ONLINE] Available at: https://pubchem.ncbi.nlm.nih.gov/compound/6324668#section=3D-Conformer. [Accessed 27 May 2021].

Rodríguez, P., Alfonso, A., Otero, P., Katikou, P., Georgantelis, D. & Botana, LM. (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(2), 1103-1111.

Yaglioglu, D., Turan, C., Erguden, D. & Gurlek, M. (2011). Range expansion of silverstripe blaasop, *Lagocephalus sceleratus* (Gmelin, 1789), to the northeastern Mediterranean Sea. *Biharean Biologist*, 5(2), 159-161.

Zhao, J., Li, G., Yi, GX., Wang, BM., Deng, AX., Nan, TG., Li, Z-H. & Li, QX. (2006). Comparison between conventional indirect competitive enzyme-linked immunosorbent assay (icELISA) and simplified icELISA for small molecules. *Analytica Chimica Acta*, 571(1), 79-85.